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Observations on the biology of *Caraphractus cinctus* Walker (Hymenoptera: Mymaridae), a parasitoid of the eggs of Dytiscidae (Coleoptera)

2. Immature stages and seasonal history with a review of Mymarid larvae

By DOROTHY J. JACKSON
North Cliff, St Andrews

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I. INTRODUCTION

For several years I have been breeding the fairy fly *Caraphractus cinctus* Walker on the eggs of various Dytiscidae. In the previous paper (Jackson, 1958*b*) I described the methods used for rearing this parasitoid, and for obtaining host eggs laid under conditions which excluded the possibility of previous parasitism. The number of imagines of *Caraphractus* bred from eggs of large and small Dytiscidae was recorded and the effects of superparasitism were described. A brief outline was given of the life history.

Although this Mymarid, formerly called *Polynema natans* Lubbock, has been

known to science for over a 100 years, the only previous observations on its biology are those given by Rimsky-Korsakov (1925), in an interesting paper in which some features of the larval life are described. The embryology of *Caraphractus reductus* R.-Kors. has been described recently by Ivanova-Kazas (1954). From the abundant material I have studied, I am now able to present an account of the various stages of this insect from the egg to the adult, and also to describe the seasonal development, which often shows remarkable instances of prolonged diapause. As far as I am aware no other description of the immature stages of an aquatic Mymarid has been published in Britain.

The Mymaridae are well-known parasitoids of the eggs of many insects. Clausen (1940) gives a useful summary of the recorded data on the morphology of the larvae, but he states that a great deal of confusion exists regarding the larval forms of the Mymaridae. *C. cinctus* has been reared from egg to adult in the egg of *Agabus bipustulatus* L. mounted in water under a coverslip. As the shell of the host egg is transparent, I have been able to observe the development of the same larvae from the early stages onwards and this has been of great assistance in studying the various larval forms.

In the course of this study I have obtained much information on the remarkable discrimination and restraint shown by the female of *Caraphratus* in laying eggs which largely prevents superparasitism. Many experiments have been carried out to determine whether the mated female can vary the sex of her offspring according to whether host eggs are offered in quick succession or at long intervals, and the results show that, on the whole, the female produces more daughters as a result of rapid laying. These matters will be discussed in a later paper.

II. TECHNIQUE

The host eggs, parasitized on known dates, were dissected at varying intervals to recover the eggs and the young larvae, dissections being made on a slide in a drop of Insect Ringer. Observations were also made on the development of the larvae by mounting host eggs in water under a coverslip and examining them by transmitted light. As the opacity of the yolk made observations difficult in the early stages of parasitism, dissections were made to obtain the eggs and young larvae. Direct observation of the living larvae in the host egg was far more satisfactory than dissection of the host for study of the later stages, because the larger larvae on removal from the host often floated upwards and burst. I usually added a drop of a very weak solution of methylene blue to the water under the coverslip containing the host egg, because this served to inhibit the growth of micro-organisms, as Balfour-Browne (1925) observed when he studied *Dytiscus* eggs.

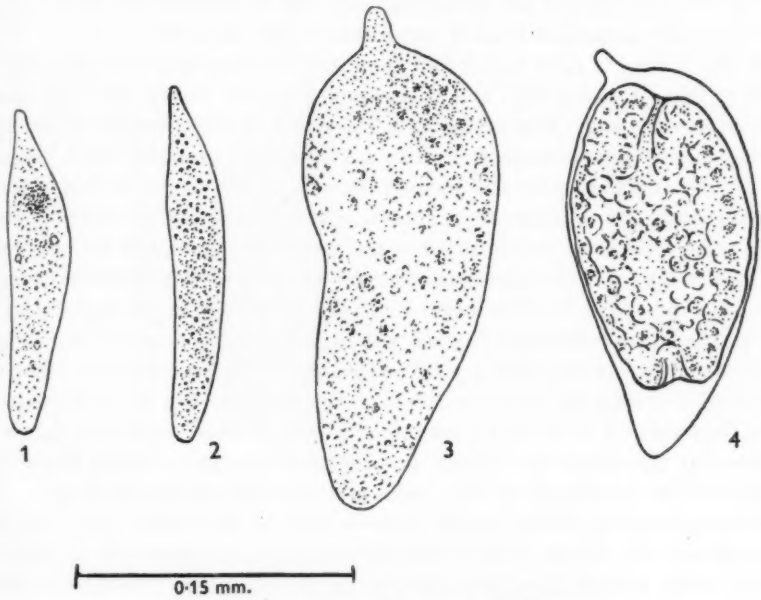
Whenever possible drawings were made of the living larvae with the camera lucida, but the larva in its second form was so continuously active that Fig. 6 is the result of several sketches. Except when otherwise stated, *Agabus bipustulatus* L. was used as host. Two or three eggs are usually deposited by *Caraphractus* as a result of one insertion of its ovipositor into the egg of *Agabus*; in superparasitism many more larvae occur, but for observations on the morphology of the larva

such crowded eggs were not used. The eggs of the host were laid in the leaves of sphagnum (Jackson, 1958*b*) and the *Caraphractus* readily laid through the leaf into the egg.

III. IMMATURE STAGES

(1) *Eggs*

The ovarian egg. When the female *Caraphractus* emerges from the host egg, numbers of eggs ready to lay are present in her ovaries and she may commence oviposition within 24 hr. of emergence. In a large female, well over 100 eggs are present. The number of eggs has been ascertained by counting the progeny reared



Figs. 1–4. *Caraphractus cinctus* Walker

All drawings made from living specimens

Fig. 1. Ovarian egg, stained with methylene blue. Fig. 2. An egg dissected from host 20 min. after laying. Fig. 3. Egg laid by the same female as egg in Fig. 2, dissected from another host, about 72 hr. after laying. Fig. 4. Egg of another female also about 72 hr. after laying, but more advanced, showing reduced size and developing embryo.

from a single female and adding to the total the eggs left in the ovaries. In this way a total of 144 was obtained from one female and 150 from another. There are two ovaries each consisting of fan-shaped clusters of eggs contained within very fine ovariole tubules which lie so closely bunched together that their number has not been ascertained. The eggs overlap each other within the ovariole tubules and the elongated nucleui of the follicular epethelium surrounding each egg show up well after staining with methylene blue. The oviduct is extremely short. From dissection of numerous females it has been found that the eggs within the same

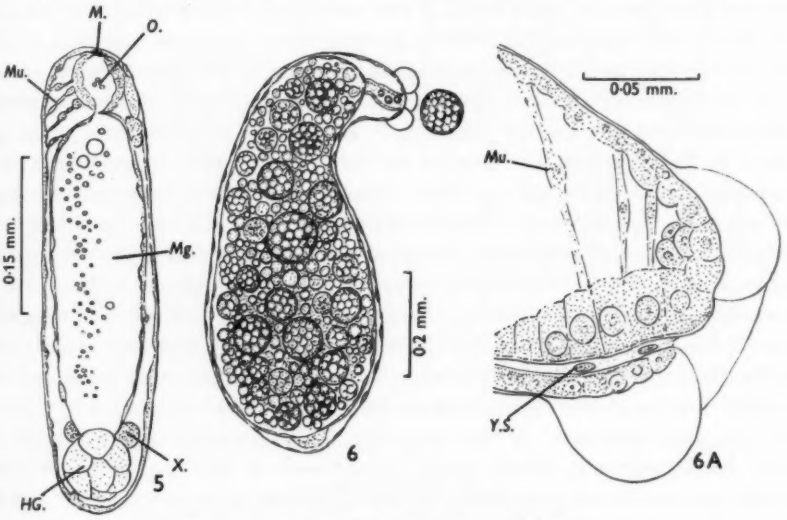
ovary vary in size. They measure from 168 by 32μ to 225 by 52μ. The ovarian egg (Fig. 1) has one side slightly concave and the other convex. The upper end (as situated in the ovary) terminates in a pedicel, the chorion of which is thicker than elsewhere. Chrystal (1930) found that in the Cynipid, *Ibalia*, the eggs are oriented in the ovary in the same way, with the pedunculated end upwards. He states that the liquid contents of the egg are forced back into the the thickened free end of the egg stalk during laying, thus leaving the egg so reduced in size that it can easily pass down the ovipositor canal. Alam (1958) stated that in the Encyrtid, *Metaphycus taxi* Alam, the egg is always found hanging by the pedicel from the puncture in the haemocoel of the Coccid host, but in *Caraphractus* the egg is placed well into the host egg and has been seen within the embryonic membrane of a host egg which was parasitized when it was already some days old.

Egg after laying. I have found that the newly laid egg recovered from the host within 20 min. of laying (Fig. 2) is narrow and elongate, rather narrower than the full-sized ovarian egg. This is no doubt the result of compression during laying. The egg increases considerably in size and especially in width after deposition (Fig. 3). In October 1956, the increase in size of the egg was determined by dissecting host eggs in Insect Ringer solution at different periods after oviposition by the same female. It was found that the egg within 20 min of laying measures 0·19 by 0·02 mm., and the largest egg recovered (3 days after oviposition) measured 0·28 by 0·1 mm. (Fig. 3). This seems to be the greatest size the egg attains, and then, as the embryo develops further, the egg appears to contract, for an egg of the same female a week after laying was smaller, 0·21 by 0·08 mm., though the embryonic development was advanced. A similar egg (Fig. 4) was obtained in March 1959 about 3 days after laying, for the rate of development of eggs is very variable. At this stage the embryo has contracted away from the lower end of the egg and the beginnings of fore-, mid- and hind-gut can be discerned.

The larva develops within the egg with its head at the anterior pole, and this is in accordance with Hallez's law (1886) regarding the orientation of the embryo in relation to the mother. The larva hatches by forcing a hole through the anterior end of the egg just below the pedicel. The newly hatched larva is surrounded by an extremely delicate membrane in which I failed to detect cellular structure, but this membrane is soon shed. When host eggs are dissected a few days after parasitism, it frequently happens that one finds squat-shaped embryo larvae surrounded by a transparent membrane, and sometimes, beside each, the crumpled remains of a hatched egg. These appear to be embryo larvae which have been prematurely forced out of their shells when the host egg was opened, as the egg contents then rush out, and larvae, when observed in the act of emerging, were of the normal elongate shape. Hatching may occur in from 3 to 9 days after laying, the eggs laid in spring and summer developing more quickly than those laid in autumn. Occasionally an egg of *Caraphractus* may fail to hatch. One was recovered in October 1956, several weeks after oviposition in an unhealthy egg of *Agabus bipustulatus*. Another was found in an egg of the same species on 12 May 1958, lying alongside a prepupa and pupa, all three resulting from oviposition by one female between 9 and 10 April 1958.

(2) *Different stages of larva*

At no period during its development is the larva clearly segmented, but indications of partial segmentation were observed in one half-grown larva that was not distended normally, like the rest, by ingested food. Sclerotic mandibles are absent in all stages and not even fleshy mandibles are discernible. No tracheae are visible in the larva and they first appear in the pupa. Throughout the larval life, as in other parasitic Hymenoptera, the mid-gut is closed behind and has no communication with the hind-gut. The waste matter is retained till the imaginal state and is not normally expelled by the imago until it has emerged from the host. Owing to the delicacy of the cuticle and the absence of sclerotic mouth parts it has not been



Figs. 5 and 6. *Caraphractus cinctus* Walker

All drawings made from living specimens

Fig. 5. First-instar larva dissected from host about 70 hr. after laying. Fig. 6, second-instar larva. Fig. 6A, head of the second-instar larva, dorso-lateral view showing oral lobes. *HG.* hind-gut; *M*, mouth; *MG*, mid-gut; *Mu*, muscle cells, extending from body wall to oesophagus; *O*, oesophagus; *X*, probably sex cells; *YS*, yolk sphere (elongated) passing down oesophagus.

possible to ascertain how many larval instars occur. The larva, however, passes through four different stages which may correspond to three or to four instars. The principal food of the larva in all its stages is the yolk spheres.

(*a*) *The first-instar larva* (Fig. 5). The newly hatched larva is slim and elongated. It is extremely transparent and only in this stage are the internal organs clearly visible. Its size varies from 0·2 by 0·09 mm. to 0·54 by 0·15 mm. The divisions of fore-, mid- and hind-gut can be distinguished readily. The larva feeds by sucking in the yolk spheres of the host's egg by contraction and dilation of its oesophagus, the movements being controlled by muscle cells attached from the oesophagus to the dorsal body wall (Fig. 5 *Mu.*). The larva at this stage is active, moving about

within the host egg by bending and then straightening its body. This slim stage appears to last only a few hours, for the larva rapidly ingests food so that the mid-gut becomes distended and packed with yolk spheres of all sizes. The shape alters quickly and the next stage is reached.*

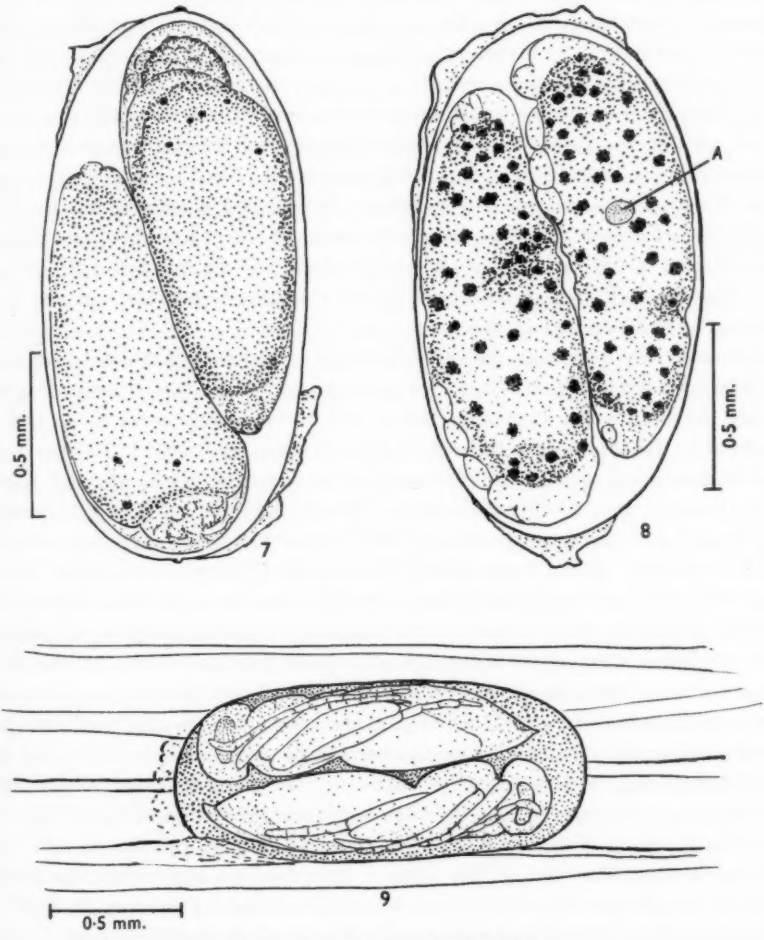
(*b*) *The second-stage larva* (Fig. 6). This is the stage in which nearly all the available food is consumed. The larva is slim in front and sack-like posteriorly, and almost the whole of its body is filled with the mid-gut. The larva is now very active, stretching out its transparent head in all directions in search of yolk spheres, and the oesophagus is in continuous spasmodic movement as the food is sucked in. The mid-gut is now packed with yolk, both in the form of globules of various sizes and also of spherical masses of globules that are larger than the mouth of the larva and may measure up to 110μ. I was puzzled to know how the larva ingested such large yolk spheres, but I have several times observed how this is done. When the larva's mouth comes in contact with one of the large spheres, as in the one at the mouth of the larva figured (Fig. 6), the larva gulps it down in portions which slide down the narrow oesophagus, and in the mid-gut these must again coalesce to form spheres. The head of the larva in this stage is provided with two transparent lateral lobes, the oral lobes, which project forwards beneath the mouth with a smaller median area between them, Fig. 6A. Rimsky-Korsakov (1925) mentions these projections in the young larva of *Caraphractus cinctus* and includes a figure of them. I believe that these projections help to guide the yolk spheres into the mouth. The larva in this active feeding stage is dark and opaque owing to the dense accumulations of yolk within the mid-gut. The larva increases rapidly in size and the contents of the gut soon become blurred and indistinct as though partially digested. Larvae in this stage measure from 0·69 by 0·12 mm to 0·9 by 0·34 mm.

(*c*) *The third-stage larva.* In this stage (Fig. 7) the larvae occupy the greater part of the *Agabus* egg and, when several are present in one egg, they are pressed together and are rounded or somewhat triangular being narrow in front and wide behind. They are now much paler as the yolk is largely digested and they are often transparent. No oral lobes have been observed in this stage. The larvae are much less active than in the preceding stage and appear to feed little. They glide slowly about changing their position relative to one another and they are constantly altering in shape. The most common shape in an uncrowded egg is shown in Fig. 7. The host egg has become opaque owing to the cloudy matter in which the larvae lie. The colour of the larva is translucent whitish with the contents of the mid-gut creamy white. The larvae appear now to have attained their full size and the next and last phase probably represents the final stage of the third instar.

(*d*) *The last-stage larva.* In the final stage the larva is somewhat bolster-like in shape being now wider at the anterior end. When two larvae are present in an egg they usually orientate themselves to face opposite ends of the egg (Figs. 7, 8).

* Ivanova-Kazas (1954) observed in the newly hatched larva of *Caraphractus reductus* a much reduced segmentation which soon disappeared. She found that the nervous system on hatching is still in an embryonic state and she considered that the first movements of feeding and locomotion are of myogenic type. In the young larva she distinguishes six ganglia of the abdominal chain.

This stage is characterized by the regular peristaltic movements of the mid-gut and by development of about 40 to 50 opaque white spots distributed all over the mid-gut wall. A few of these spots, while still small, were observed in the posterior region of the younger larvae (Fig. 7). The spots consist of large single cells situated



Figs. 7–9. *Caraphractus cinctus* Walker

All drawings made from living specimens

Fig. 7. Egg of *Agabus bipustulatus* L. with two third-stage larvae of *C. cinctus*, about 9 days after laying. The opaque spots of the mid-gut wall are few and restricted to the posterior end. Fig. 8. Egg of *A. bipustulatus* containing two full-grown larvae of *C. cinctus*; *A*—discoloured spot in the shell of *Agabus* egg formed round the oviposition puncture. Fig. 9. Egg, probably of *A. sturmii* Gyll. (on leaf of *Juncus articulatus*) containing two newly formed pupae of *C. cinctus*; the upper is that of a male, the lower is that of a female.

Figs. 8 and 9 are according to D. J. Jackson previously published in *J. Soc. Brit. Ent.* 1956 and 1958, by kind permission of Dr E. J. Popham.

in the wall of the mid-gut and each one is densely packed with spherical concretions which are probably products of excretion. The concretions vary in size from 1 to 18μ in diameter and the smallest (1–2μ) exhibit Brownian movement. These cells with concretions are widely scattered in an apparently structureless membrane constituting the mid-gut wall. Intervening cells in the gut wall were not evident in stained whole mounts, and the structure of the mid-gut epithelium requires further investigation.* I formerly believed (Jackson, 1958*a*) that the larvae which did not show the spots were in a younger instar, but having watched their gradual appearance in the same larva on several occasions without any moult being apparent, I have no doubt that the deposition of these concretions represents the final phase of digestion in the full-grown larva. Feeding has been completed before the concretions appear and before the marked peristalsis of the mid-gut sets in. This forward and backward movement of the gut no doubt promotes the absorption of nutriment and the waste products will be deposited in the concretions. The fat-body makes its appearance in the larva before the concretions form and is quite independent of them.

No mouth parts have been distinguished in the full grown larva the general shape of which is shown in Fig. 8. The larvae vary considerably in size according to the number present in one host egg. When two larvae are present in an egg of *A. bipustulatus* the size varies from 1·11 by 0·29 mm. to 1·36 by 0·47 mm. Apart from examination of the mid-gut and its conspicuous white spots I have not studied the internal anatomy of the larva. Rimsky-Korsakov refers to the presence of Malpighian tubes, but Ivanova-Kazas (1954) states that they are not formed in *Caraphractus reductus*. In the Pteromalid, *Mormoniella* (*Nasonia*) *brevicornis* Ashmead, Tiegs (1922) did not find Malpighian tubules in the larva, but he describes three digestive glands which he calls 'hepatic caeca' opening into the mid-gut posteriorly.

The complete absence of the tracheal system from all stages of the larva of *Caraphractus* is interesting. Ivanova-Kazas also noted the absence of a tracheal system in the larva of *C. reductus* Rim.-Kors. According to Clausen (1940, p. 117) several authors emphasized the complete lack of a tracheal system in the larvae of *Trichogramma*. Presumably the larva of *Caraphractus* must absorb oxygen cutaneously, in its early stages by way of the host tissues, and in the last instar, when the contents of the egg have been consumed, through the shell of the egg from the surrounding water. Yet when I immersed an egg containing last-stage larvae in liquid paraffin, the larvae were still alive and moving 18 days later. Whiting (1940) observed that when eggs of the Ichneumonid, *Habrobracon juglandis* were placed in the mineral oil 'Nujol', development proceeded normally.

* It is interesting to note that Ivanova-Kazas (1952, p. 194) found that the mid-gut wall of the newly hatched larva of *Prestwichia aquatica* Lubbock is composed of about 30 very large cells, the enlargement of the gut being due to the growth of the cells and not to their increase in number; it is accompanied by a great stretching of the cell walls, so that in later stages of development the gut wall appears as a structureless membrane, the boundaries of the cells being invisible. In the wall of the mid-gut of the first-instar larva of *Caraphractus*, when examined in optical section, I have noticed that the cells only show at long intervals, with apparently structureless membrane connecting them, just as was shown by Ivanova-Kazas (1954) in her figure of a longitudinal section of the young larva of *Caraphractus*.

(*e*) *Larval habits.* The larvae in all stages of growth have been observed alive in the eggs of various Dytiscidae but they have never been observed to attack each other. Moreover, it often happens that a small larva in a crowded *Agabus* egg will attain the imaginal state while the larger specimens in the same egg remain as larvae. Sometimes a dead larva is found in an egg of *A. bipustulatus* or *Ilybius fuliginosus* after the other parasites have completed their growth or even emerged, and in *Dytiscus* eggs residual larvae are common.

(3) *Prepupa*

When the larva has finished feeding it enters into a resting stage, the prepupa which is distinguishable from the mature larva by the absence of the peristaltic movements of the mid-gut. The two extremities of the larva have become transparent, the mid-gut having contracted from either end. The head has become larger and the opaque spots in the mid-gut have come closer together owing to the contraction of the mid-gut. In spring and summer the prepupal stage normally lasts for only a few days, but very rarely individuals may enter into diapause and spend a prolonged period as prepupae. The prepupae formed during the late autumn normally remain throughout the winter in this stage within the host egg.

Just before the pupal ecdysis a churning movement takes place in the mid-gut, the opaque concretions begin to coalesce and the larval cuticle is shed to form a small crumpled mass at the apex of the abdomen.

(4) *Pupa*

The pupal state is easily recognized by the disappearance of the white spots which were so striking a feature of the mature larva and the prepupa. Soon after ecdysis all the solid waste matter forms a compact yellowish white mass lying within the pupal abdomen. There can be little doubt that this waste matter is largely derived from the concretions which will be liberated into the lumen of the pupal intestine by the break-down of the epithelium of the larval mid-gut (Jackson, 1958*a*). This opaque matter within the gut occurs in some pupae in the anterior part of the abdomen, and in others posteriorly, depending on the position of the pupa. The solid waste matter is not normally discharged until the imago has emerged from the host egg, when it is shed as the meconium, as observed by Rimsky-Korsakov (1925) and Jackson (1958*a*). It consists largely of numbers of minute concretions just like those in the cells of the wall of the mid-gut of the larva. In these cells as well as in the imaginal discharge, these concretions, which are doubly refractile when seen in polarized light with crossed nichols, are possibly urate concretions. Exceptionally, when the imago is unable to emerge, the meconium is shed within the host egg. At other times the imago may die within the host egg with the abdomen distended and the meconium undischarged. Such bloated specimens may result when too few imagines develop in a *Dytiscus* egg.

The fact that, in *Caraphractus*, the waste matter accumulated during the larval life is not normally shed until the imago emerges, indicates that the mid- and hind-gut are not in communication until the adult stage is reached. A similar late

discharge of the meconium has been observed by Flanders (1942) in *Trichogramma*, for he states that the absence of a meconium showed that the egg of the codling moth had been parasitized by a species of *Trichogramma*, whilst the presence of meconial pellets showed that a *Prospaltella* sp. had developed within the egg. There is thus variation in egg parasitoids in regard to the stage at which defecation occurs. In the Scelionid, *Hadronotus ajax* Girault, which develops in the egg of the squash bug *Anasia tristis* De Geer, Schell (1943) notes that the larva discharges the meconium after the formation of the cocoon within the egg of the host, and in *Prestwichia aquatica* Lubb., Henriksen (1922) observed that the excrement was deposited in the host egg at pupation. In the Mymaridae there is surprising variation. In *Erythmelus* and *Alaptus*, Bakkendorf finds that the excrement is evacuated before the transition to the pupal stage, and in *Polynema* at the transition to the pupal stage. This is confirmed by Balduf (1928) for *Polynema striaticorne* Girault. With *Caraphractus* on the other hand the excrement is always voided by the imago.

The pupa of *Caraphractus* is at first colourless except for the mid-gut which is yellowish white. Tracheae are present and show best in the antennae and legs. In this colourless stage the sexes can be most readily distinguished by the antennae, for in the female they are clubbed and short, barely reaching the abdomen, while in the male they are long and filiform, reaching half way down the abdomen (Fig. 9). The eyes of the pharate imago are the first part to darken. They become at first pink and then turn to black. The ocelli are also pink before darkening. The dark coloration of the adult appears later in the thorax and legs. The head at this time is strangely parti-coloured, with a dark sclerite on the face between and below the eyes and a small dark bifid plate at the back of the head, surrounding the ocelli. The whole of the vertex is in strong contrast being colourless and transparent. At this stage the abdomen shows dusky dorsal bands and the ovipositor becomes dark. In a few days the body acquires the normal adult coloration and the pupal cuticle is cast. The imagines at first have the abdomen pale and the vertex of the head transparent. They remain for a day or two within the egg and may move about, changing their position, but mating has never been observed within the host egg. The wings are fully developed before emergence and the antennae are completely freed from the pupal integument while the imago is still in the egg. Crandell (1939), however, notes that in the Pteromalid, *Pachycrepoideus dubuis* Ashmead, a parasite of the pupal state of the fly *Piophila casei* L. the imago pulls off the antennal sheaths with the forelegs after emergence from the puparium. Strangely enough, Matheson & Crosby (1912) record that a male of *Caraphractus cinctus* was seen to emerge from an egg of *Notonecta* and then strip off the pupal sheaths from antennae and legs. This is entirely contrary to what I have observed in breeding *Caraphractus* in eggs of Dytiscidae. The egg vacated by *Caraphractus*, if it has been parasitized at an early stage, is left completely empty except for a little crumpled heap of the larval and pupal exuviae.

IV. EMERGENCE FROM HOST EGG

The number of holes made by the imagines emerging varies according to the size of the host egg. In the eggs of *Agabus* and *Ilybius* in which two to three imagines usually develop, these fill up the egg, lying parallel to each other and to the long axis of the egg. In such eggs normally one emergence hole is made near the end of the egg and, in an egg with six imagines, all emerged from the one hole. In the egg of *Dytiscus marginalis* many *Caraphractus* may develop, and the imagines, after shedding the pupal cuticle, face in all directions. It is a feature of the parasitism of these large eggs that in most cases all the parasites do not reach maturity at the same time, even if the egg has been exposed to only one female for 24 hr. The imagines which develop may have difficulty in emerging, their movements being impeded by a mass of immobile larvae and pupae. In superparasitized *Dytiscus* eggs the imagines may die without emerging, together with all the larvae and pupae. In the *Dytiscus* egg from two to five irregular holes are normally made for emergence and these are variable in position.

The process of emergence has been watched under a binocular microscope in eggs of *Agabus*. Before emergence the abdomen in both sexes is somewhat distended by the unshed meconium, and shows pale intersegmental bands. When both sexes were present in an egg it was the male that cut the emergence hole. One male succeeds in penetrating the shell with its mandibles and tugging at it, the antennae lying backwards and the ventral surface of the body being pressed closely to the egg. It continues to bite at the shell until a rift appears and a narrow sliver of the shell is cut off and falls into the egg. Sometimes when two or more males are present they may work together to enlarge the same hole, removing successive small strips. When many males are present in the same egg all trying to get out, one jostles the other out of place, so that gnawing at the shell is interrupted and emergence is delayed. When one or two females develop alone in an *Agabus* egg they make the hole in the same way. Directly the imagines emerge they shed the meconium as a string-like mass of chalky white matter. The males, which usually emerge first, remain on the egg watching for another imago to emerge and they do this even when only males are present in the egg. They mate instantly and successively with the females as they emerge. If no females emerge, the males pursue each other with the tip of the abdomen bent downwards. It is only the newly emerged males that are attractive to other males and they soon lose interest in each other. Faure (1926) observed the same behaviour of males towards a newly emerged male in *Pimpla instigator* F. As the egg of *Agabus* is often surrounded by plant tissue, such as a sphagnum leaf, the imagines bore their way through this also, and those which parasitize the eggs of *Ilybius* and *Dytiscus* embedded in plant tissue, either emerge through the scar left by the beetle on laying eggs, or cut a hole in the leaf above the egg. Although the imagines which emerge from host eggs into the water have no air bubbles upon them, their trachae are filled with air.

V. EFFECTS OF PARASITISM ON HOST EGGS OF DIFFERENT AGES

Recently laid host eggs are the most suitable for the development of *Caraphractus*, for in such eggs all the contents are available as food for the larvae. When newly laid eggs of the smaller water beetles, *Agabus* and *Ilybius*, are exposed to a single attack by one female, three eggs are most frequently deposited before the ovipositor is withdrawn, but often two eggs are laid or occasionally only one. Even if only one parasite develops in such an egg all the contents are devoured, and a large specimen, usually a female, emerges. When three eggs are laid, one male and two females are usually reared, all of good size. A newly laid egg of *Dytiscus* can provide food for from twenty-five to thirty-five *Caraphractus* larvae as the result of many ovipositions by one female, or of several layings by a few females. If sufficient larvae are not present in these large eggs, there is too much food available and physiogastric specimens develop which are incapable of breeding (Jackson, 1958*b*).

Eggs of *Agabus* which are advanced in development may be parasitized even when the host larva is already formed, but in such eggs rarely more than one or two parasites develop. In *Dytiscus* eggs, on the other hand, it is rare to obtain any living parasites when an advanced egg has been exposed to the attack of *Caraphractus*. Usually, in such cases, both host egg and parasites die. In one *Dytiscus* egg with a well-developed embryo a single male *Caraphractus* emerged and one physiogastric female, while four full-grown larvae and one male pupa were left in the egg. Such advanced eggs are unattractive to females if at the same time newly laid eggs of *Dytiscus* are available.

(1) *Parasitism of newly laid eggs of* Agabus bipustulatus

Parasitism does not cause the immediate death of the host egg. After oviposition by the Mymarid in a newly laid egg of *Agabus* the normal formation of large yolk spheres subsequently occurs, just as it does in an unparasitized egg. These large rounded or polyhedral spheres which show in the egg of *Agabus* a few days after laying would appear to be homologous with the polyhedral masses shown in the figure of the egg of *Clytra* reproduced by Imms (1957, fig. 169), resulting from a secondary cleavage of the yolk. It would appear that the development of the host egg continues normally until the *Caraphractus* larva hatches. Thus if an *Agabus* egg parasitized in October, not more than 2 days after it had been laid, was dissected a week later, the parasite egg had not hatched and the host embryo had developed; but when a newly laid host egg is parasitized in summer the *Caraphractus* larva hatches in about 3 days and the host embryo does not form. One cannot distinguish a parasitized from an unparasitized egg during the first few days of parasitism, except that after a variable time of from 1 to 4 days the puncture hole becomes red, showing in optical section as a red plug in the vitelline membrane, and the puncture eventually turns black. The vitelline membrane constitutes the principal part of the shell of the *Agabus* egg, for the outer membrane, the chorion is a fragile, readily ruptured membrane (Jackson, 1958*c*). If the egg has been exposed to many females numerous punctures may be made and the dark dots may lie

so close together that they form blotches. When the *Caraphractus* egg hatches, the young larva is able to move freely amongst the yolk spheres on which it feeds.

(2) *Parasitism of advanced eggs of* Agabus bipustulatus

When the host embryo is already formed at the time when *Caraphractus* oviposits the development of the parasite may be followed more clearly than in the newly laid egg, owing to the absence of the yolk spheres from the periphery, for these are now enclosed in the mid-gut. Such eggs have therefore been used largely in my study of the larval stages and for observing oviposition. The behaviour of the female when laying eggs in advanced eggs of the host differs in some respects from her attacks on newly laid eggs. The ovipositor is thrust in more deeply in order to penetrate into the mid-gut of the host, where it appears that the eggs are usually deposited, amongst the yolk spheres. The mid-gut of the host lies nearest to the back of the egg and therefore is most easily reached when the female stands on the back of the leaf, for the *Agabus* egg is usually laid in such a position that the back of the egg rests against the inside of the leaf. When the host egg is newly laid, the female may attack it successfully at any spot without a deep insertion of the ovipositor. When a female attacks an advanced egg from the head end or the ventral side, she may puncture the egg repeatedly for short trials until she finally holds her ovipositor in for the usual time required for laying, and may then plunge it in so deeply that her body almost touches the leaf or the egg. The first signs of successful parasitism of an advanced egg are the movements of the young *Caraphractus* larvae within the mid-gut of the *Agabus* embryo where they feed upon the yolk spheres. There seems little doubt that the stimulus for deposition of the egg will be afforded by the consistency of the contents encountered by the ovipositor, and that the presence of yolk granules or spheres dispersed throughout the host egg when newly laid, and later grouped into polyhedral masses, and finally present as large spheres within the mid-gut, affords a suitable fluid medium in which to release the eggs.

The host embryo is not paralysed as it may move its body and legs after parasitism. It continues for a few days to develop normally and the pale mandibles may become sclerotic. The parasitic larvae had been at first found in the mid-gut, but later, in the course of their movements, they usually rupture the mid-gut dispersing the large yolk spheres within the body of the host, even into the head and the coxal cavities. As the parasitic larvae increase in size, the legs and appendages of the host, if they are not already sclerotic, shrink and become transparent, the hypodermis receding from the cuticle, and finally they disintegrate and are consumed by the larva. The breakdown of the host tissue makes the egg opaque until the larva has finished feeding, when it again becomes clear. Thus, in eggs parasitized at an early stage of embryonic development, no host tissue is left. When, however, the host larva is fully formed at the time of oviposition, the hard parts, unsuitable as food for the larvae, persist. One may thus find, eventually, one or two pupae of *Caraphractus* lying in the area formerly occupied by the mid-gut and surrounded by the cuticle of the host, with head, mandibles, mouth parts,

legs and cerci intact. Occasionally the pupae may be found surrounded by a cellular membrane representing the remains of the epithelium of the mid-gut of the host. The parasitism in an advanced host, owing to a more restricted food supply, is not always successful. Often the host larva partially emerges and dies, or else succumbs with the parasites within the egg.

(3) *Darkening of shell of parasitized eggs*

Once the larvae of *Caraphractus* are full grown, the vitelline membrane of the host egg (*Dytiscus*, *Ilybius* or *Agabus*) becomes brownish, which is due to the deposition of numerous minute dark dots. This darkening of the host shell is known for various egg parasitoids. Salt (1933) notes the the host egg parasitized by *Trichogramma evanescens* Westwood turns black when the larva is full grown and Balduf (1928) observed that the vitelline membrane of the egg of the hopper *Ceresab ubalus* Fabr., turned black when advanced larvae or pupae of *Polynema striaticorne* Girault were present. Ferrière (1926) notes that the eggs of Psocids parasitized by *Alaptus minimus* (Hal.) Walk. became violet with metallic reflections. Quednau (1957) attributes the darkening of host eggs parasitized by *Trichogramma cacoeciae* March. to the death of the vitelline membrane.

VI. THE EGGS AND LARVAE OF OTHER MYMARIDAE

(1) Eggs

According to Clausen (1940) there is little variation in the form of the egg in the Mymaridae but the length of the pedicel is variable. Thus in *Polynema striaticorne* Gir., figured by Balduf (1928), the pedicel is as long as the rest of the egg, but in *Caraphractus* the pedicel is much shorter and the egg is very like that of *Patasson* (*Anaphoidea*) *nitens* Gir. as figured by Tooke (1953). Ison, 1959, notes the remarkable length of the egg of *Anagrus ensifer* Debauche which he states 'equals more than one fourth that of the normal fairy fly'.

(2) Larvae

(*a*) *Classification*

In his important paper Bakkendorf (1934) provides a synopsis of the known Mymarid larvae and it will be of interest to consider how far the larva of *Caraphractus* fits into his classification. In his synopsis Bakkendorf recognizes only two larval instars. He classifies the larvae into two groups; The group I is characterized by the presence in the second instar of two cephalic outgrowths below the mouth, which he considers as the first pair of legs. He stated that the first-instar larva (at that time only known for *Anagrus*) is bag-shaped with clumsy cephalic and caudal parts. In this first group Bakkendorf places *Anagrus*, *Erythmelus* and, strangely enough, *Caraphractus*. No cephalic outgrowths comparable with legs have been observed in any larval instar of *Caraphractus* that I have examined, for the oral lobes of the second-stage larva are more like a protruding labium than legs. The first-instar larva of *Caraphractus* was unknown to Bakkendorf when he compiled his synopsis and it is entirely different from the first-instar larva of

Anagrus, hence neither the young nor the mature larva of *Caraphractus* fits into Bakkendorf's group I.

In the second group Bakkendorf includes *Polynema*, *Alaptus*, *Lymaenon*, *Ooctonus*, *Patasson* (*Anaphoidea*) and *Mymar* but the identity of the larva described by Bakkendorf as ? *Mymar pulchellus* has now, he informs me, been established as *Enaesius* sp. In this second group the first-instar larva has the cephalic part with a frontal process above the mouth (which may persist into the second instar) and it has a slender tapering caudal appendage. It is evident that the first-instar larva of *Caraphractus* does not fit into this group either.

Clausen (1940) recognizes two types of first instar Mymarid larvae, (1) the sacciform type represented by *Anagrus* (and also by *Prestwichia*, but this genus is correctly included in the Trichogrammatidae by Kloet & Hincks, 1945 and so is omitted here) and (2) the mymariform type as present in *Polynema* and other genera. The first-instar larva of *Caraphractus* does not conform to either of these types.

(*b*) *Comparison of the larva of* Caraphractus *with other Mymarid larvae*

In its first instar, the larva of *Caraphractus* is characterized by its elongate cylindrical shape and the absence of mandibles or cuticular projections, in the second stage by the presence of oral lobes and in the last instar by its bolster-like shape and conspicuous opaque internal spots. The larvae of the other genera show many differences which will be briefly described here and summarized in the Table 1.

(1) *Mymarid genera*

Anagrus. The newly hatched larva is sac-like with a posterior constriction. It has been figured by Henriksen (1922) for *A. brocheri* Schulz (the species is stated by Bakkendorf, 1925, to be *incarnatus* Hal.), while Henriksen (1922) and other writers consider that figures given by Ganin (1869) of the larval stages of a '*Polynema*' refer to a species of *Anagrus* as was previously discussed (Jackson, 1958*b*). Dumbleton (1934) described the first-instar larva of *Anagrus armatus* Gir. as an inactive ovoid body, constricted posteriorly. During further development are formed the cephalic appendages, mandibles and paired ventral outgrowths of the hind-end of the body. This second stage is well figured by Bakkendorf (1934) for *A. incarnatus* and he stated that its fat cells assume a deep red colour in this instar. Bakkendorf's illustration of *A. incarnatus* is reproduced here as Fig. 10. This stage has been described by Ganin as 'histriobdellid' which according to Henriksen is peculiar to *Anagrus*. Debauche (1948, p. 40) stated, erroneously, that *Caraphractus* also has a histriobdellid larva, no doubt following Bakkendorf's classification (1934, p. 93).

Paranagrus. The figure given by Perkins (1905) of the head in the first-instar larva of *P. optabilis* Perkins, shows an elongated frontal process and a finger-like process below. No ventral outgrowths from the hind-body are shown in his figures and he did not observe a histriobdellid stage. In *P. osborni* Fullaway, the newly hatched larva was described by Fullaway (1918) as a soft apodous grub-like segmented and hairless larva provided with long slender chitinized mandibles

Table 1. *Mymarid larvae*

Type of larva	Species	First instar	Later instars	Host eggs	Reference
First instar sometimes sacciform, later instar histriobdellid	*Anagrus incarnatus* Haliday = *A. brocheri* Schulz.	Sacciform, head end lobate, separated from body by constriction	Second instar histriobdellid. Mandibles and finger-shaped cephalic outgrowths. Hind-body with paired ventral outgrowths. Henriksen reports last instar only with mouth-hooklets	Coenagriidae; *Erythromma*; *Agrion*; Delphacidae; *Conomelus*	Henriksen, 1922; Bakkendorf, 1925, 1934; Ganin ('*Polynema*'), 1869
	Anagrus atomus L.	'Early larva' (possibly second instar) with curved mandibles and beneath them a pair of appendages. Body segmented with paired ventral outgrowths on sixth segment	Similar to early larva. Posterior appendages disappear prior to pupation	Typhlocybidae; *Typhlocyba*	Macgill, 1934
	A. epos Girault	Not recorded	Half-grown larve histriobdellid	Typhlocybidae; *Typhlocyba*	Mulla, 1956
	A. bartheli Tullgren	Not recorded	Curved mandibles and two membranous lobes beneath them	Typhlocybidae; *Typhlocyba*	Tullgren, 1916
	A. armatus, var. *nigroventris* Girault	Unsegmented ovoid body with rounded caudal tubercle	Body segmented, anal segment with two ventral processes	Typhlocybidae; *Typhlocyba*	Dumbleton, 1934
	Anagrus sp.	Not recorded	Curved mandibles and two fleshy protuberances at each side of mouth	Delphacidae; *Delphax* (*Liburnia*)	Enock, 1914
	Paranagrus optabilis Perkins	Not recorded	No histriobdellid stage observed. Head of full-grown larva with frontal process and papilla	Delphacidae; *Perkinsiella*	Perkins, 1905
	P. osborni Fullaway	With long slender chitinized mandibles, moving between slender papillae. Body hairless segmented	Not recorded	Delphacidae; *Perigrinus*	Fullaway, 1918

Table 1 (*cont*).

Table 1 (*cont*).

Type of larva	Species	First instar	Later instars	Host eggs	Reference
First instar, 'mymariform'. Body with long hairs	*Polynema pusillum* Haliday	Long frontal process, hook-shaped mandibles. Body segmented, with long hairs and long caudal process	Hairless, with small frontal process and large mandibles	Jassidae	Bakkendorf, 1934
	P. euchariforme Haliday	Similar to *P. pusillum*	Similar to *P. pusillum*	Bathyscopidae; *Idiocerus*	Bakkendorf, 1934
	P. ovulorum Haliday	Similar to *P. pusillum*	Similar to *P. pusillum*	Typhlocybidae; *Cicadella*	Bakkendorf, 1934
	P. striaticorne Girault	Long and short frontal processes. Body segmented, with long hairs. Caudal appendage	Bag-like with cephalo-ventral prominence with falcate mandibles	Membracidae; *Ceresa*	Balduf, 1928
	Enasius sp.	Frontal process short, caudal appendage with teeth at base; body with long bristles	Not recorded	Heteroptera?	Bakkendorf, 1934
	Anaphes nipponicus Kuwayama	Short frontal process, long caudal appendage. Body with many long hairs	Bag-like without appendages or hairs	Chrysomelidae; *Lema*	Kuwayama, 1935
	Patasson (*Anaphoidea*) *nitens* Girault	Curved frontal process. Body segmented, with many spines and long caudal appendage	Indistinct segmentation, small cephalic process	Curculionidae; *Gonipterus*	Tooke, 1953; Clark, 1931
	P. (*Anaphoidea*) *calendrae* Gahan	Body with at least four setae and caudal appendage	No setae or caudal appendage	Calandrinae; *Calandra*	Satterthwait, 1931
	P. (*Anaphoidea*) *conotracheli* Girault	Unknown	Disciform, no outgrowths, mandibles not observed	Chrysomelidae. Dytiscidae; *Agabus*	Bakkendorf, 1934
First instar simplified mymariform, hairless	*Ooctonus heterotomus* Foerster	Short frontal process, and mandibles. Body segmented, hairless, with caudal appendage	Cylindrical, segmentation not defined, minute mandibles and finger-shaped frontal outgrowth	Hemiptera	Bakkendorf, 1934
	Lymaenon effusi Bakkendorf	Frontal process and mandibles. Body segmented, hairless, with caudal appendage	Mandibles present	Jassidae; *Aphrodes* (*Acocephalus*)	Bakkendorf, 1934
	Alaptus minimus (Hal.) Walker	Mandibles present. Body fusiform, hairless with tapering caudal extremity	Mandibles not seen	Psocoptera	Bakkendorf, 1934
	Erythmelus goochi Enock.	Unknown	Powerful mandibles, two cuticular outgrowths at mouth, segmental folds laterally	Heteroptera	Bakkendorf, 1934
First instar elongate. Second with oral lobes	*Caraphractus cinctus* Walker	Elongate, cylindrical, no mandibles or outgrowths, no visible segmentation	Second instar with oral lobes. Last instar bolster-like, widened in front	Dytiscidae; *Agabus*, *Ilybius*, *Dytiscus*, *Colymbetes*, *Hydroporus*. ? Notonectidae; *Notonecta* in U.S.A. (Matheson and Crosby, 1912)	Jackson, 1958*a*, *b*; Rimsky-Korsakov, 1925; Ivanova-Kazas, 1954

moving between slender papillae. These species are of considerable economic importance in the control of leaf-hoppers in Hawaii, and *P. optabilis* is of interest in being capable of thelytokous parthenogenesis for several generations (Perkins, 1905).

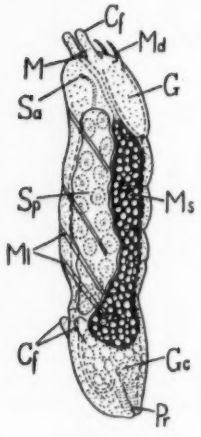


Fig. 10

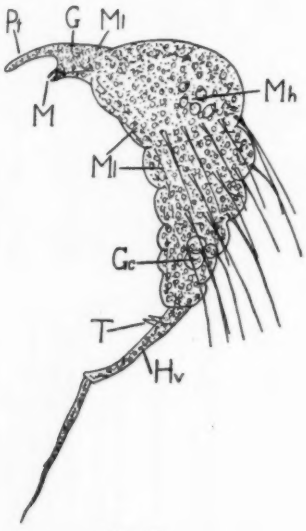


Fig. 11

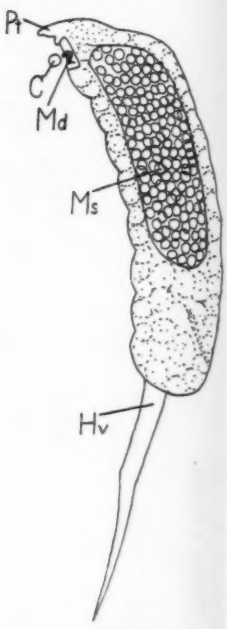


Fig. 13

Fig. 14

Fig. 12

Fig. 15

Figs. 10–15. For legend see opposite page.

Polynema. The mymariform first instar larvae of various species have been described, and Bakkendorf's figure of *P. pusillum* Hal. (Fig. 11) is typical of the genus. The frontal process of the head, and the segmented body bearing long hairs are characteristic of the genus as is the caudal appendage. This varies in length and in shape in the different species being much shorter in *P. striaticorne* Gir. as figured by Balduf (1928) than in *P. pusillum* and *P. euchariforme* Hal. as shown by Bakkendorf. After the first moult the prominent apendages are reduced and the hairs are lost, but Bakkendorf records the presence of mandibles in the second-instar larva of *P. pusillum*.

Enaesius. The first-instar larva was figured by Bakkendorf (1934) as (?) *Mymar pulchellus*, but, as already noted, he later identified it as *Enaesius* (Fig. 12). It may be considered as mymariform.

Anaphes. The larva of *A. nipponicus* Kuwayama has been described by Kuwayama (1935) and it is typically mymariform.

Patasson (Anaphoidea). The first-instar larva of *P. nitens* is also mymariform. Tooke (1953) made the interesting discovery that the male and female larvae of this species differ markedly in the first instar and can also be distinguished in the second instar. In the first instar the male larva has fewer spines than the female larva and the cauda has a different shape, while in the next instar the sexes can be distinguished by the shape of the cephalic process.

Ooctonus. Bakkendorf has described the larva of *O. heterotomus* Förster in its different stages, including the first instar (Fig. 13). The larva in its later stages resembles that of *Caraphractus*, except that small mandibles are present. Like *Caraphractus* the segmentation is not defined. Balduf (1928) suggested that failure to locate segmentation in Mymarid larvae may be due to the improper dilution of salt in a medium used to study the insects. This cannot apply to *Caraphractus* since numerous larvae studied alive within the host egg, immersed in water, showed no segmentation, except one empty young larva, previously described (cf. p. 5).

Lymaenon and Alaptus. The larval stages of *L. effusi* Bakkendorf and *A. minimus*

Figs. 10–15. Mr O. Bakkendorf's figures

Fig. 10. *Anagrus incarnatus* Haliday. Second larval instar before the appearance of the 'symbiotic' cells; oblique lateral view, × 94.

Fig. 11. *Polynema pusillum* Haliday. First larval instar, × 188.

Fig. 12. *Enaesius* sp. First larval instar, × 282.

Fig. 13. *Ooctonus heterotomus* Förster. First larval instar, × 188.

Fig. 14. *Lymaenon effusi* Bakkendorf. First larval instar, × 188.

Fig. 15. *Alaptus minimus* (Hal.) Walker. First larval instar, × 188.

Explanation of lettering of Mr Bakkendorf's figures

C, yolk sphere; *Cf*, cuticular processes of the cephalic and caudal parts; *G*, brain; *Gc*, reproductive cells; *Hv*, caudal appendage; *M*, mouth; *Md*, mandibles; *Mh*, stomach cavity; *M*1, muscles; *Ms*, stomach with contents of undissolved yolk spheres; *Pr*, proctodaeum; *Pt*, frontal process; *Sa*, orifice of salivary duct; *Sp*, salivary gland; *T*, tooth.

(Hal.) Walker were described by Bakkendorf (Figs. 14, 15). Like the larvae of *Ooctonus* they are hairless.

Erythmelus. The later larval stages of *E. goochi* Enock were described by Bakkendorf. The youngest larva observed was cylindrical and characterized by the waggling sideways motions which are performed by powerful lateral segmentally arranged muscles.

(2) *Anatomical differences in Mymarid larvae*

As it has already been mentioned (cf. p. 10) the excrement in different genera may be voided at different stages of the parasitic life. The long, paired salivary glands described by Bakkendorf in several genera (e.g. *Anagrus*, *Polynema*, *Lymaenon* and *Ooctonus*) appear to be completely absent from the *Caraphractus* larva. Rimsky-Korsakov figures, in the side view of the larva of *C. cinctus* a small globular 'salivary' gland behind the oesophagus. In this position I have found a similar body in the first-instar larva, but I hesitate to name it. Ivanova-Kazas (1954) figures a short salivary gland opening below the mouth in the young larva of *C. reductus*.

There is also, according to Bakkendorf, variation in regard to the presence or absence of the cells with concretions in the mid-gut wall of the larva, which Bakkendorf (1934) regards as symbiotic cells. He observed them in *Anagrus*, *Lymaenon* and *Ooctonus*, but he states that these cells appear to be absent from *Erythmelus*, *Polynema* and *Alaptus*. Perkins (1905) shows a few dark spots in his figure of the larva of *Paranagrus optabilis* Perk. in the same position as the cells with concretions in the *Caraphractus* larva.

(*c*) *Scarcity of records*

From the data so far available it is clear that there is remarkable variation in the structure of the larvae of the Mymaridae. As Clausen (1940) pointed out the greatest diversity occurs in the young larvae; the older larvae of the various genera show a general resemblance. While numerous species of Mymaridae have been used for the control of a great variety of insect pests in various parts of the world, in many cases no account has been given of the larval morphology of the species concerned, while in other cases the larvae were inadequately described and in some genera the larval form appeared to be quite unknown. Species of the genus *Gonatocerus* (Mymaridae) are recorded from a variety of insect eggs, but I have failed to find any description of the larvae. Dr Hincks, however, informed me that it would be better to use the name *Lymaenon* instead of *Gonatocerus* since he considered that the latter name is probably incorrectly applied (Kryger, 1934). It has already been noted that Bakkendorf has described the larva of *L. effusi*. Bak.

(*d*) *Arrangement of the Table*

An attempt has been made to collect the information available and to present it in tabular form to show the principal features of the larval anatomy in the different forms of Mymarids. It will be seen that the *Polynema* group with hairy,

mymariform, first-instar larvae, is well defined, as is the 'histriobdellid' stage in the development of *Anagrus*. The genera, *Ooctonus*, *Lymaenon* and *Alaptus* have first-instar larvae of mymariform shape, but they are hairless; these larvae are termed here 'simplified mymariform'. *Caraphractus cinctus* so far forms a type of its own.

In the table, the host genera are recorded only as they are given by the authors cited; many other hosts are known for some species. Hincks (1950) in his valuable paper includes an extensive list of hosts of British Mymarid genera and in his later paper (1952) he gives an account of recent literature on the family. The nomenclature I have adopted is that approved of by Dr Hincks.

VII. SEASONAL HISTORY

I have bred *Caraphractus cinctus* from eggs of *Agabus*, *Ilybius* and *Dytiscus* collected at various seasons of the year in ponds in Fife, and there can be little doubt that this Mymarid parasitizes the eggs of the various species of Dytiscidae as they become available throughout its breeding season. In the many breeding experiments which I have carried out the females laid readily in the eggs of the different species offered to them (Jackson, 1958*b*), irrespective of the host from which they themselves had been bred. There is no evidence to suggest that there may be biological races adapted to certain hosts. If such specialization did occur, it would be practically impossible for one strain to breed continuously throughout the season, for the period of laying of the different host beetles is usually restricted to several weeks of the year (Jackson, 1958*c*). *Agabus bipustulatus*, however, has a long laying period extending from autumn till spring, but even in the summer a few laying females have been obtained, and this species has therefore proved of great value for carrying out experimental breeding of *Caraphractus*.

During 1956 this Mymarid was reared on a succession of host eggs of different species, and, as this probably represents the natural sequence of hosts available in any small pond, it will be of interest to record the data. At the end of March 1956, I reared two females of *Caraphractus* from an egg of *Ilybius fuliginosus* parasitized the previous July and also four males from an egg of *Agabus bipustulatus* parasitized in early October. From matings between these, breeding of *Caraphractus* was commenced. The first generation was reared on eggs of *A. bipustulatus* and of *A. sturmii* Gyll. and the imagines emerged from the middle to the end of May. The next generation was bred on eggs of the same *Agabus* spp. and also on the eggs of *Colymbetes fuscus* L. and *Dytiscus marginalis*, which were then obtainable. It is during May, when host eggs are most abundant, that the Mymarid is likely to start increasing in great numbers. The imagines of the second generation of *Caraphractus* emerged from mid-June till the beginning of July. They laid in the eggs of *A. sturmii* and *D. marginalis* and by the end of June in eggs of *I. fuliginosus* which were also available. The third generation of parasites emerged from these eggs principally from mid-July till the end of the month, but some did not emerge till the latter part of August. As the eggs of *A. sturmii* were then unobtainable and those of *Dytiscus* were very scarce, breeding was continued on the

eggs of *I. fuliginosus*, and the fourth generation of *Caraphractus* emerged from the middle to the end of August, by which time the eggs of *A. bipustulatus* were again available. The imagines of the fifth generation began to emerge in the middle of September and in October (when they laid in eggs of *A. bipustulatus*), but only a portion of the fifth generation emerged before the winter. The majority hibernated as prepupae in host eggs of *A. bipustulatus* and *I. fuliginosus* to emerge the following year at variable dates mostly from March till May.

While four full generations and a partial fifth have thus been bred in one year in an unheated north room, there is much overlapping of generations, because, even in the spring and summer, there is great variation in the time of emergence of different individuals from host eggs parasitized at the same time. Mating may sometimes occur between individuals of different generations. My experiments show that, when the specimens are kept in a cool room, there is a continuous breeding of this insect from March till October. Hamm (1917) captured imagines in water at Oxford as late as 27 October. Occasional specimens, bred entirely in captivity, or reared from collected host eggs brought indoors some weeks previously, have emerged during the winter, but it is very doubtful if this would occur out of doors. That this Mymarid is probably inactive in ponds during the colder weather is suggested by the fact that eggs of *Dytiscus marginalis* collected in water-lily pools in Fife on 12 March 1957, were all unparasitized, while eggs of the same species collected in the same pools in the early summer were heavily parasitized by *Caraphractus*, especially in 1955 (Jackson, 1956). This *Dytiscus* begins to lay eggs very early in the year and it seems probable that the first laid eggs will escape parasitism.

In the hot summer of 1959 six generations of *Caraphractus* were reared in an unheated north room. Breeding started in early March and the last generation emerged in early October. The period of development from egg to imago takes nearly 6 weeks in spring and early summer, but, in 1959, during the latter part of July and in August, the period was only 23 days. Solitary specimens in an *Agabus* egg are large and take a few days longer to develop than when two or three are present. The small specimens in overcrowded eggs develop faster. In an incubator at 23° C. the life cycle was completed in 18–19 days, and the females so bred were fertile. Rimsky-Korsakov (1925) concluded that in north Russia there are three generations in the year.

(1) *Diapause*

Caraphractus passes the winter as a diapausing prepupa within certain host eggs. Some specimens may, however, go into prolonged diapause in the prepupal stage and an imago has been reared in July from an egg of *Agabus bipustulatus* parasitized the previous September. Development of some specimens has continued slowly for many months and a live imago was found in one egg of *A. bipustulatus* more than 16 months after oviposition by *Caraphractus*. Sometimes one imago will emerge from an *Agabus* egg at the normal time and the other *Caraphractus* in the same egg will emerge some weeks later, but more often the residual specimens die. When in diapause this Mymarid shows some resistance to drought. Even if the host egg

dries up and collapses the prepupae may resume development when replaced in water. They can also survive for days in freezing water.

(2) *Increase in numbers*

The females are able to start laying within 24 hr. of emergence and large mated females may lay well over 100 fertile eggs the greater proportion of which develop into females. Provided some host eggs suitable for parasitism are available, large females may live about 10 days, spent in searching for eggs to lay in. The possible increase in numbers with five generations in the year is thus immense. The high rate of parasitism amongst eggs of Dytiscidae collected in ponds has already been recorded (Jackson, 1956, 1958*c*). Breeding experiments have shown that when sufficient host eggs are available and when these are supplied singly to a female and are removed directly oviposition has taken place, the parasite suffers little or no mortality. Thus in one experiment in which 45 eggs of *A. bipustulatus* were used in early July 1958, 94 specimens of *Caraphractus*, 24 males and 70 females were reared from one female, once mated, and in only three host eggs the parasites failed to develop. In another experiment, with one female of *Caraphractus* in August 1957, using 54 host eggs, 121 imagines of *Caraphractus* were bred, 31 males and 90 females, and only two host eggs became unhealthy. When host eggs are scarce, superparasitism may occur. This affects the fertility of the parasite by the production of small females (Jackson, 1958*b*), and, often, in overcrowded eggs all the parasites die.

(3) *Other hosts of* Caraphractus

I have not yet obtained any evidence that eggs other than those of Dytiscidae can be parasitized by *Caraphractus*. My attempts to rear this species on eggs of dragonflies and Heteroptera such as *Notonecta* proved unsuccessful (Jackson, 1958*b*). The females of *Caraphractus* repeatedly punctured the eggs of *Notonecta glauca* L., but usually withdrew the ovipositor within only 5–10 sec. of the insertion, and not a single *Caraphractus* was reared from the many *Notonecta* eggs exposed. Yet Matheson & Crosby (1912) record rearing *Caraphractus cinctus* from eggs of *Notonecta* at Ithaca. The specimens which were sent to Lord Avebury for identification were seen by Mr Fred Enock, who considered them identical with British *C. cinctus*. Dr Hincks informed me that he had recently re-examined, at the British Museum, the fragments said to have been reared from *Notonecta* eggs, and that he found a wing which he felt certain was that of a species of *Caraphractus*. If the host egg was correctly identified, it would thus appear that *Notonecta* is a host of *Caraphractus* in America. Rimsky-Korsakov (1925) records *Caraphractus* reared only from eggs of Dytiscidae in Russia. The possibility of other hosts cannot, however, be ruled out, and it is interesting to find that in another Mymarid, *Anagrus incarnatus* Hal., Bakkendorf (1925) observed that the winter is passed in the larval state within the eggs of various Homoptera deposited in *Juncus*, but, in summer when the eggs of these species are not available, Agrionid eggs in water-lily leaves, as well as Homopterous eggs in other plants may be parasitized.

VIII. SUMMARY

The immature stages of *Caraphractus cinctus* Walker are described.

Four larval stages have been observed which may correspond to three or four instars. In no stage have mandibles been observed.

The first-instar larva is elongated and active. In the second-instar oral lobes are present. In the next stage the larva becomes shapeless and rather transparent. In the last stage the wall of the mid-gut shows conspicuous opaque spots which consist of single cells containing spherical concretions, probably the products of excretion.

These concretions later become free in the gut of the pupa and are discharged in the meconium when the imago emerges from the host egg.

The effects of parasitism on the host eggs of different ages is discussed. In newly laid host eggs all the contents are consumed by the parasitic larvae. In advanced eggs of *Agabus* the egg of *Caraphractus* is laid in the mid-gut of the host embryo where the parasitic larva develops, the hard parts of the host larva remaining intact. Parasitism in advanced eggs of *Dytiscus* is rarely successful.

The larvae of other Mymaridae are discussed and their main features compared. The first-instar larva of *Caraphractus* does not correspond to any of the previously described types of mymarid larvae.

The seasonal history is described, with the sequence of hosts parasitized throughout the year. From four to six generations of *Caraphractus* have been bred indoors during the year. The winter is passed as a diapausing prepupa. In some cases diapause may be prolonged. Fertility is high: 121 imagines have been bred from one female of *Caraphractus*.

I have much pleasure in acknowledging my gratitude to the late Dr W. D. Hincks for his kindness in reading the typescript of this paper: the comments by such a well-known authority on Mymaridae have been most valuable. I am very grateful to Mr O. Bakkendorf for allowing me to reproduce several of his figures of Mymarid larvae from the valuable work he devoted to the biology of Danish hymenopterous egg-parasites. I am also grateful to Miss B. A. Trott of the Commonwealth Institute of Entomology for the help she has so kindly given me with references.

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Some observations on the free-living stages of *Ostertagia ostertagi*, a stomach worm of cattle

By J. H. ROSE

Ministry of Agriculture, Fisheries and Food Central Veterinary Laboratory, Weybridge

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INTRODUCTION

Parasitic gastro-enteritis is considered to be the most widespread of the economically important helminth diseases of British cattle. It is surprising, therefore, to find that it has not been the subject of major research in this country. In recent years, a limited amount of work has been done in other parts of the world, particularly in America where the epidemiology and treatment of the disease have been studied by a number of workers, but the biology of the gastro-intestinal nematodes which cause parasitic gastro-enteritis has received little attention.

Invariably several species are involved in natural infections. In this country at least seventeen species are known, but the pathogenicity of the different species is not fully understood, and there is no reliable information regarding their incidence.

From routine post-mortem examinations of calves which have died from the disease, it does appear, however, that certain species are more prevalent than others. One of these is the stomach worm *Ostertagia ostertagi*.

A study of both the parasitic and the free-living stages of this nematode is being carried out at Weybridge, one aspect of the work being concerned with the bionomics of the free-living stages. The present paper is an account of observations on these stages in their natural environment, and of experiments carried out in the laboratory.

METHODS

Outdoor observations

Observations were made on the rate of development of the free-living stages at different times of the year, and on the migratory activities and longevity of larvae in faeces, on herbage, and in soil.

Rate of development

Faeces, collected from the rectum of an artificially infected, stalled calf, were deposited, in the form of a pat, onto a plot of perennial rye-grass growing in a galvanized iron box, and kept out-of-doors. This was repeated at the beginning of each month for 1 year. Samples of faeces were examined each week from the pats which had been deposited on plots from November to March, preliminary observations having indicated that development was likely to be slow at this time

of the year. Pats deposited outside during the remainder of the year were examined daily, as it was anticipated that the rate of development would increase with a rise in temperature. Separation of the different free-living stages from the faeces was effected by salt flotation and centrifugation (Michel & Rose, 1954). After the larvae had reached the third stage, samples of faeces were examined each month to determine the length of time that larvae remained in the pat. The time taken for the pat to disintegrate completely was also noted.

The migratory activities and longevity of larvae in the faecal pat

Two series of observations were carried out starting at the end of August 1957 and the beginning of June 1958. Both were terminated in August 1959.

Faeces, collected from artificially infected, stalled calves, were thoroughly mixed to ensure, as far as possible, an even distribution of eggs. Those used in the first series of observations contained approximately 225 eggs per gram, but those used in the second series contained only 50 eggs per gram. Two pounds of faeces were deposited in the form of a pat, 6 in. in diameter and 1 in. thick, on to the centre of a plot of perennial rye-grass, 18 in. square, growing in a galvanized iron box and kept out-of doors. The grass was approximately 2 in. in height at the outset. Replicates were set up on a number of plots. One of the replicates was sampled each week for the first 8 weeks; subsequently sampling was at intervals of 4 weeks.

Samples of faeces were taken from the centre, edge and intermediate parts of the pat (Fig. 1) using a cork borer, thus obtaining a sample through the depth of the pat. Each sample was thoroughly mixed and a 2 g. subsample was examined for eggs and larvae. Additional 2 g. samples were taken in replicate at three different vertical levels, namely 'top', 'middle' and 'bottom'. The 'top' level corresponded to the crust covering the faecal pat, and was approximately $\frac{3}{16}$ in. in depth. The 'middle' and 'bottom' levels were each approximately $\frac{7}{16}$ in. in depth. In this way it was possible to study both the lateral and vertical distribution of larvae in the pat, and to assess their migratory activities. As the faecal pats disintegrated fewer samples were taken.

The migratory activities and longevity of larvae on herbage and in soil

Having sampled the faeces on a plot, the herbage was then removed and examined, in the first series of observations each week for the first 8 weeks, then at intervals of 4 weeks for the remainder of the first year, and at intervals of 8 weeks during the second year. In the second series, examinations were made at intervals of 4 weeks throughout. In both series the herbage was removed in the morning between 9 and 10 a.m.

The herbage on the plot was divided laterally into three areas designated 'inner', 'outer I' and 'outer II' (Fig. 2). In each of these areas it was also divided vertically by first cutting at a level 2 in. above the soil surface to give the 'Upper' herbage, and then at soil level to give the 'Lower' herbage, so that altogether six samples were obtained from each plot. By examining these samples separately, it was possible to study the lateral and vertical distribution of the larvae, and to

assess the extent and direction of migration. Recovery of the larvae from the herbage was effected by a process of washing and sedimentation (Michel & Rose, 1954). The larvae, in three samples, each of 5 ml. of the final washing, were counted, and the total number of larvae on the different herbage samples estimated. Occasionally there was an excessive amount of debris in the final washings and it was necessary to separate the larvae by salt flotation and centrifugation; the larvae in three 5 ml. samples of the salt solution were then counted.

The upper $\frac{1}{2}$ in. of soil was removed from each of the areas and larvae separated and enumerated using the method described by Rose (1955).

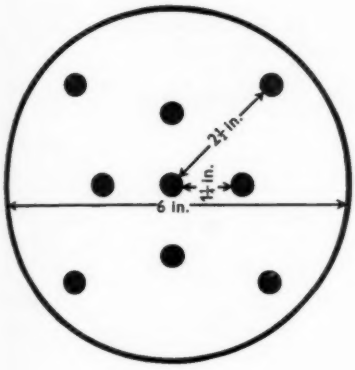

Fig. 1

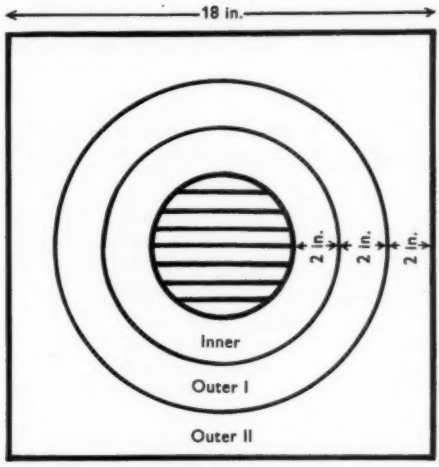

Fig. 2

Fig. 1. Lateral sampling of faecal pat. The shaded circles represent the areas from which samples of faeces were taken using a cork borer.

Fig. 2. Lateral sampling of herbage. Plan of grass plot showing the division into three areas for lateral sampling.

Laboratory experiments

Laboratory experiments were carried out to assist in the interpretation of the outdoor observations. The effect of temperature on the rate of development of eggs and larvae in faeces, and the effect of temperature and humidity on the longevity of eggs and larvae, separated from faeces, were studied. The methods used were almost identical with those described in detail in a previous paper dealing with similar observations on the larvae of *Dictyocaulus filaria* (Rose, 1955). They differed in one respect in that some of the present experiments were concerned with the eggs of the nematode. In order to determine whether the eggs were alive after being subjected to a particular experimental treatment, they were incubated for several days at 27° C. and a relative humidity of 100 %. Viability was then assessed from the numbers of first-stage larvae which hatched.

RESULTS

The rate of development varied considerably according to the time of year, being much quicker during the summer than during the winter (Table 1), when development more or less stopped. A small proportion of the eggs deposited out-of-doors in December had developed into second-stage larvae by the end of February, and a small proportion of those deposited out-of-doors in January became embryonated within a few weeks, but most of the eggs in faeces deposited outside in December, January and February, underwent little development during

Table 1. *The rate of development of the free-living stages of* Ostertagia ostertagi *in faeces deposited out-of-doors at different times of the year*

Infected faeces deposited out-of-doors in	Time taken for development to third-stage larva (weeks)	
	Minimum	Maximum
1957		
September	2	5
October	3	5
November	8	11
December	11	20
1958		
January	8	17
February	8	13
March	6	8
April	3	4
May	2	3
June	1	2
July	< 1	2
August	< 1	2

the winter. Although there was some mortality, appreciable numbers of eggs survived to complete their development into third-stage larvae in the spring. It was assumed that these variations in the rates of development were largely a result of the different temperatures prevailing (Fig. 3). This assumption was supported by the laboratory experiments, which clearly demonstrated that the rate of development was influenced by temperature, being slowed down as the temperature decreased (Table 2).

The rate of development was also influenced by other factors. There were appreciable individual variations at all times of the year. This was particularly pronounced during the winter, when there was as much as 9 weeks difference in the time taken for eggs, deposited out-of-doors at the same time, to develop into third-stage larvae (Table 1). These individual variations were associated with the position of the eggs in the faecal pat; those nearest to the surface generally developed more quickly than those in the depths of the pat. A probable explanation is that the inner part of the pat was initially less well aerated than the outer part, for it is known from laboratory experiments that aeration influences development. Eggs suspended in aerated water develop more rapidly than those in non-aerated

water. Even when the eggs are all subjected to the same conditions, there is, however, a small amount of individual variation in the rate of development.

Although the eggs were distributed more or less evenly throughout the pat, the majority of the third-stage larvae were recovered from the upper surface layer designated 'top' (Table 3), indicating a marked vertical migration by these larvae. They were recovered from this region even when it was hard and dry, while the internal part of the pat was moist and soft. It was evident from the numbers of

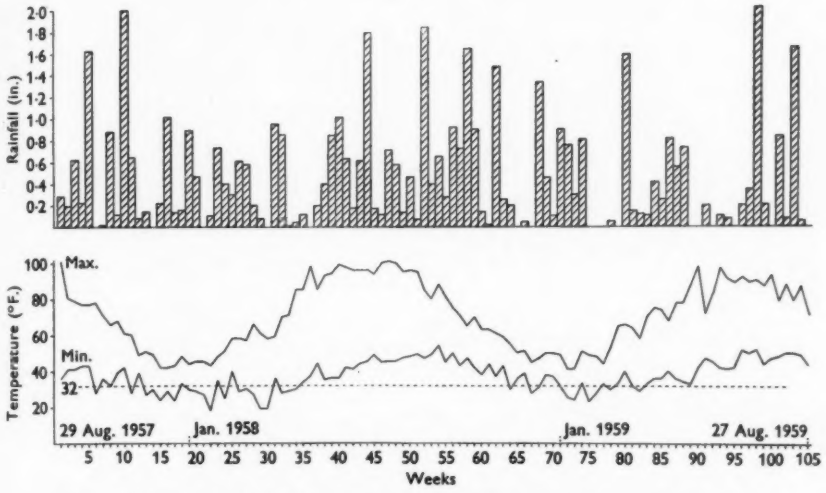

Fig. 3. Mean weekly minimum and maximum ground temperatures, and total weekly rainfall from 29 August 1957 to 27 August 1959.

Table 2. *The effect of temperature on the rate of development of the free-living stages of* Ostertagia ostertagi *in moist faeces*

Temperature (°C.)	Time taken for development to third-stage larva (days)	
	Minimum	Maximum
22–23	3	7
14–16	7	16
10–11	18	28

Table 3. *Numbers of third-stage larvae recovered from 2 g. samples of faeces taken at different levels of the faecal pat.* (*Four typical results are tabulated.*)

Date of sampling	No. of third-stage larvae from		
	Top	Middle	Bottom
26 September	521	31	13
3 October	340	1	16
10 October	218	14	0
17 October	147	12	0

larvae recovered from the herbage that a marked lateral migration had taken place, but this was not so clearly evident from the lateral sampling of the pat.

Most of the third-stage larvae eventually migrated on to the herbage, but small numbers were recovered from the faeces until the pat had disintegrated completely. The time taken for this to happen varied considerably (Table 4).

Table 4. *The time taken for the complete disintegration of faecal pats deposited out-of-doors at different times of the year, and the length of time during which larvae were recovered from these pats*

Infected faeces deposited out-of-doors	Time for complete disintegration of faecal pat (months)	Length of time during which larvae were recovered from faecal pat (months)
1957		
September	7	6
October	9	8
November	7	6
December	7	6
1958		
January	7	6
February	7	6
March	11	10
April	7	6
May	11	10
June	13	12
July	13	12
August	13	12

Migration on to the herbage was a gradual process. In the first series of experiments, development to the third stage took between 2 and 3 weeks. A small number of larvae were recovered from the herbage soon afterwards, but it was several weeks before the herbage infestation reached a high level (Fig. 4). At the same time, there was a corresponding decrease in the numbers of larvae recovered from the faecal pat (Fig. 5). The same pattern of migration was observed in the second series of experiments.

The majority of the larvae did not migrate far from the faecal pat, being recovered from the 'Inner' area (Fig. 6). Some did migrate to the outermost part of the plot ('Outer II') that is, they moved between 4 and 6 in. away from the pat, but the numbers recovered were so small that they have not been recorded separately. They have been combined with those from the 'Outer I' area, which constitute the greater proportion of the larvae represented in Fig. 6, under the heading 'Outer' area.

A proportion of the larvae recovered for each of the three areas of the plot was from the 'Upper' herbage (Fig. 6), indicating that vertical migration had taken place to a limited extent. It was particularly interesting to observe that living larvae were recovered from the 'Upper' herbage at all times of the year, even during the unusually hot and dry summer of 1959. By far the greatest numbers were

recovered from the 'Lower' herbage, however. The numbers recovered from the soil were negligible.

Third-stage larvae survived for a surprisingly long time. The maximum observed longevity on herbage out-of-doors was almost 2 years (Fig. 4), but in the laboratory,

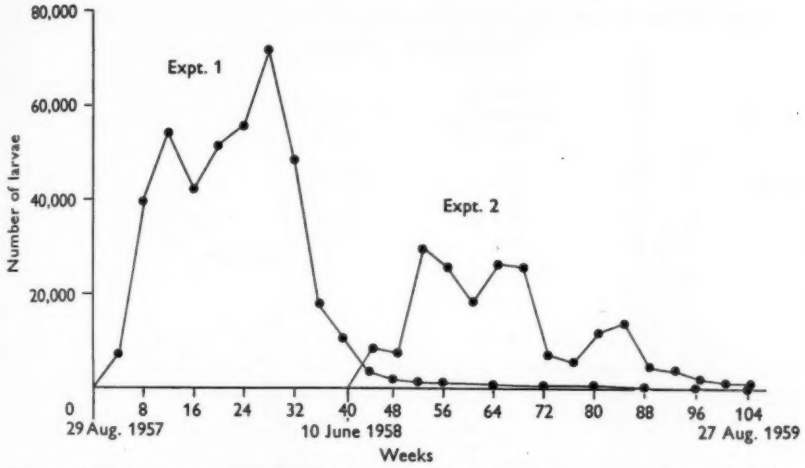

Fig. 4. Longevity of larvae on herbage. Each point represents the total number of larvae recovered from all of the herbage of a single plot.

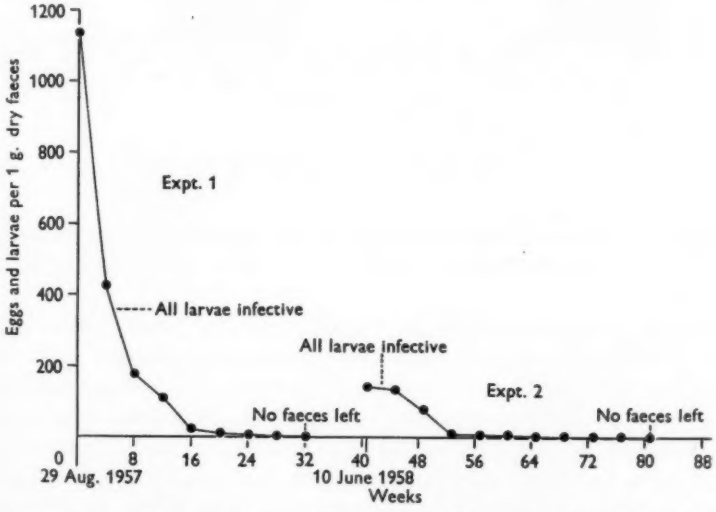

Fig. 5. Persistence of larvae in faeces. The number of larvae per 1 g. of dry faeces was estimated from the mean of all of the samples taken from a single pat.

larvae in water survived even longer at low temperatures (Table 5). Only a small proportion of the larval population survived for these long periods of time, but on the grass plots large numbers of third-stage larvae survived for almost

8 months (Fig. 4). Climatic factors did not appear to have any marked effect on their longevity. Many survived throughout the winter (Fig. 4) which suggests that they have a good degree of tolerance to low temperatures. This was confirmed

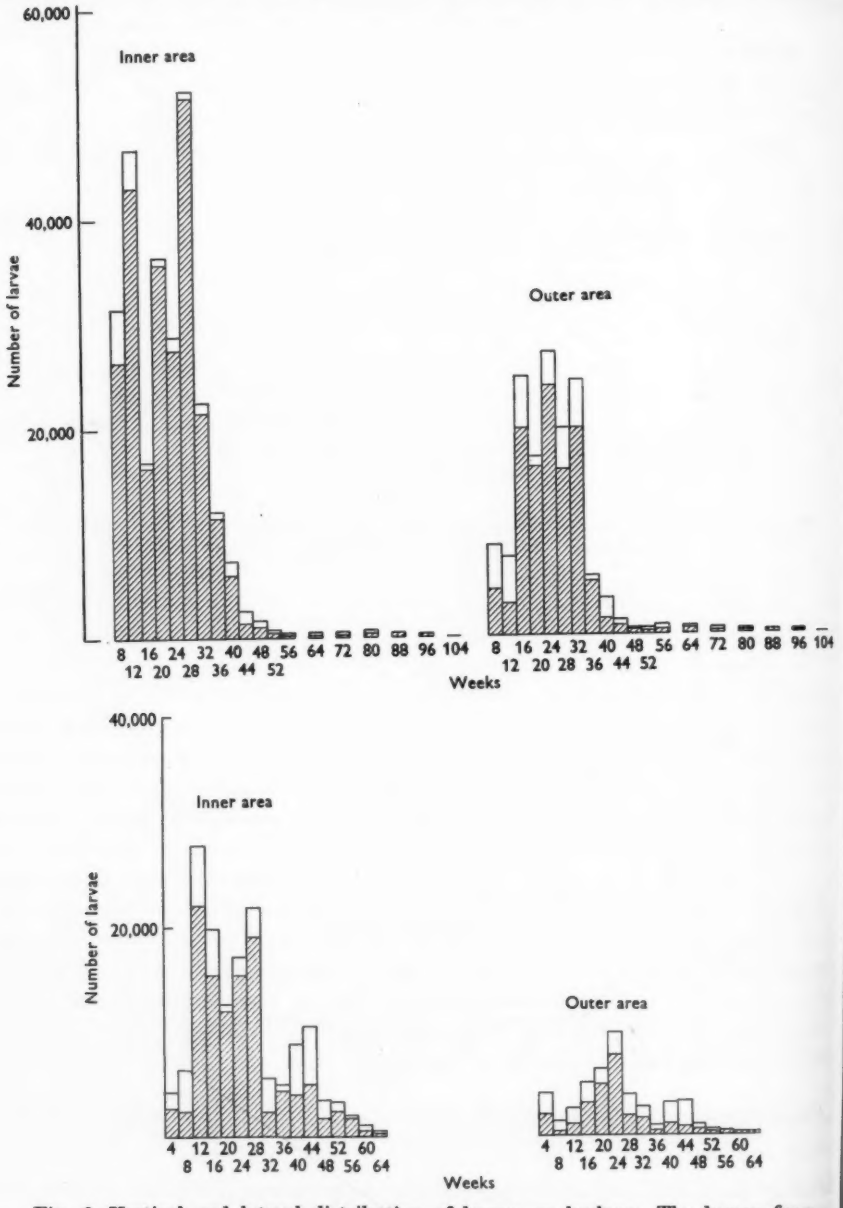

Fig. 6. Vertical and lateral distribution of larvae on herbage. The larvae from the 'Outer II' areas have been plotted as a single histogram under heading 'Outer area'. □ = 'Upper' herbage; ▨ = 'Lower' herbage.

by the laboratory experiments which demonstrated that eggs as well as third-stage larvae were able to withstand continuous freezing for several weeks, but first- and second-stage larvae were very susceptible, being unable to survive for 24 hr. (Fig. 7).

Table 5. *The maximum longevity of infective larvae in water kept at different temperatures*

Temperature (°C.)	Maximum longevity (weeks)
24–25	29
15–16	55
10–11	109
6–7	134

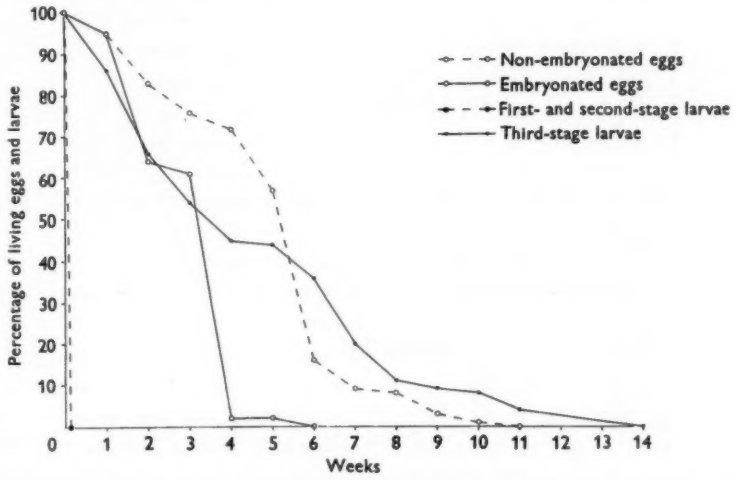

Fig. 7. The effect of freezing on the longevity of eggs and larvae. The eggs and larvae suspended in water were subjected to a temperature of $-4°$ to $-6°$.

In the second series of experiments many larvae survived throughout the summer, thus suggesting a good degree of tolerance to high temperatures, which was confirmed in the laboratory experiments, in which third-stage larvae survived for a maximum of 29 weeks at 24–25° C. (Table 5), with less than 1 % mortality during the first 8 weeks. Unfortunately the summer of 1958 was fairly wet and it was not possible to observe the effects of natural dry conditions until the summer of 1959 (Fig. 3), by which time the larval population on the herbage had decreased. Even so, larvae were shown to survive during the hot and dry months of 1959, thus suggesting a good degree of resistance to dryness. This was further demonstrated in the laboratory experiments (Fig. 8). Embryonated eggs also were able to survive for a short time under dry conditions, but non-embryonated eggs and first and second-stage larvae were extremely susceptible to dryness.

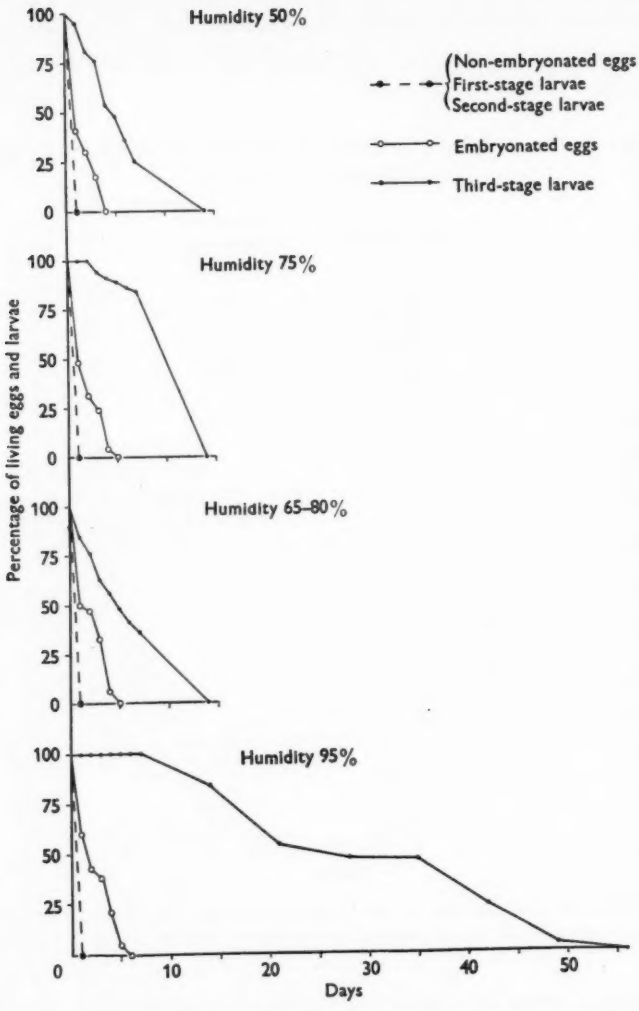

Fig. 8. The effect of desiccation on eggs and larvae. At 100% humidity, 92% of third-stage larvae were still alive after 56 days.

DISCUSSION

From the present observations it is possible to obtain a general idea of how translation (that is, the process whereby eggs in faeces become infective larvae on herbage available to the grazing bovine) will operate in the field in areas having climatic conditions comparable with those studied.

Survival and development of the eggs and larvae will depend to some extent on the form in which faeces are deposited on the pasture. Faeces deposited as compact pats afford a considerable degree of protection from the external climatic conditions,

and eggs and larvae in them can survive under climatic conditions which would otherwise prove fatal. In a pat, development will generally be completed at all times of the year, except during the winter when the temperature will be too low. Although there will be some mortality it is not likely to be excessive, even during the winter. The eggs which overwinter can continue their development in the spring as the temperature rises. If infected faeces are deposited on a pasture throughout the winter, there will, therefore, be the possibility of a build-up of infection on the herbage in the spring.

When faeces are disseminated over the pasture in some way, either as a result of their consistency when excreted, or by the intervention of mechanical agencies, the eggs and developing larvae will be more exposed to the external climatic conditions than when they are protected in a faecal pat. Survival and development may then be adversely affected. From the laboratory experiments it can be seen that mortality is likely to be increased during the winter as a result of the unfavourable effects of freezing, and during dry periods as a result of the extreme susceptibility of eggs and first- and second-stage larvae to dryness.

This effect of dryness was well illustrated in experiments carried out during the summer of 1959 (to be described in detail in the future), when none of the eggs in spread faeces succeeded in developing into third-stage larvae.

Migration of the third-stage larvae on to the herbage appears to depend upon the condition of the faecal pat, which is in its turn influenced by the climatic conditions. The pats deposited out-of-doors in August 1957 and June 1958 remained fairly soft and moist. From time to time the upper surface tended to dry out, but rain was so frequent that it was soon moistened and softened again. Some larvae migrated on to the herbage soon after becoming infective and the majority had left the pat by the end of 4 months. In the course of other experiments, faecal pats were deposited outside in May 1959 at the start of the dry summer (Fig. 3). The upper surface was hard and dry within a few days and gradually the entire pat dried out and remained so for almost 4 months. During this time there was no appreciable decline in the numbers of third-stage larvae in the pats, and only very small numbers were occasionally recovered from the herbage. Later in the year, after rainfall had softened the pats, the larvae were able to migrate on to the herbage. It seems that dry conditions can be regarded as unfavourable to migration from the faeces, whereas wet conditions are favourable. At all times the faecal pat will serve as a reservoir of infection for several months.

In the field there will not always be this time lag between the larvae reaching the third stage and migrating on to the herbage. The faeces themselves may be disseminated over the pasture by various agencies, thereby assisting the larvae to reach the herbage more quickly.

For the third-stage larvae to be effective in the transmission of infection they must be situated on herbage that will be ingested by the grazing bovine. It would appear that only a small proportion of the larvae will be in the most favourable situation for this to take place, that is on the 'Upper' part of the 'Outer' herbage (Fig. 6). The majority of the larvae recovered from the experimental plots were on the 'Inner' herbage, which corresponded to the tussock of herbage around the

pat frequently avoided by the grazing bovine, so that the chances of the larvae being ingested would not be very good. It is, however, impossible to predict accurately which larvae will be ingested, as the grazing behaviour of the bovine is very variable. It is well known that when keep is short the bovine is far less selective in its grazing than when it is plentiful, and it will then ingest even the tussocks of herbage around pats.

In considering the various stages involved in translation, it can be seen that, in general, little translation will occur during the winter in view of the limiting effect of low temperatures on development. For the remainder of the year, when the temperature will generally be conducive to development, translation will be influenced to a considerable extent by the amount and frequency of the rainfall, being facilitated under wet conditions and limited by dry conditions.

Although the present experiments were concerned essentially with only one stage in the transmission of infection, that is translation, it is possible to draw some tentative conclusions about the transmission of infection in the field.

It is logical to suppose that the transmission of infection will take place most readily when conditions are conducive to translation, but in view of the longevity of the third-stage larvae it should be possible for transmission to be effected at all times of the year.

The longevity of the third-stage larvae is of particular practical significance. Various American workers including Baker (1939), Goldberg & Rubin (1956), Drudge, Leland, Wyant & Rust (1958), have demonstrated by field experiments that infection can survive on pastures for up to 9 months. The present observations suggest that infection can persist for considerably longer, at least in southern England. It is unlikely, however, that the small proportion of larvae which survive for 1 year or more are of much importance. Far more important are the many larvae which can survive for 8 months or so on the herbage. This means that a pasture heavily infected in the autumn and rested over the winter may still be heavily infected in the following spring when young stock are turned out. It is also a factor of great importance in relation to control by grazing management. Much present-day advice is based on the assumption that the larval population on a pasture decreases rapidly in the absence of reinfection. This assumption often appears to be without adequate foundation, as the bionomics of the free-living stages of most of the species of bovine gastro-intestinal nematodes have never been studied. As the present observations have shown, it is not true of the larvae of *Ostertagia ostertagi*. Much work remains to be done before it is known whether this applies to the larvae of the other species of gastro-intestinal nematodes associated with bovine parasitic gastro-enteritis, and before control by grazing management can be formulated on a sound basis.

SUMMARY

Outdoor observations were made on the rate of development of the free-living stages of *O. ostertagi* at different times of the year, and on the migratory activities and longevity of the larvae in faeces, on herbage and in soil.

The effect of temperature on the rate of development of eggs and larvae in faeces, and the effect of temperature and humidity on the longevity of eggs and larvae when separated from faeces, were studied in the laboratory.

The results of these observations are discussed in relation to translation (that is, the process whereby eggs in faeces become infective larvae on herbage available to the grazing animal) and to the transmission of infection in the field.

I wish to thank Mr J. F. Michel for providing the infected faeces, and Mr D. A. Griffiths for technical assistance.

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Studies of the amino acids of *Rhodnius prolixus*

I. Analysis of the haemolymph

By J. S. HARINGTON

*Department of Entomology, London School of Hygiene and Tropical Medicine**

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The high amino acid concentration in the haemolymph of insects is a biochemical characteristic of this class of animal (see Florkin, 1949). It is much higher than the levels of free amino acids in human plasma, and among other things plays an important role in osmoregulation (Bishop, Briggs & Ronzoni, 1925; Beadle & Shaw, 1950). Studies of haemolymph amino acids have been greatly extended by the technique of paper partition chromatography (Consden, Gordon & Martin, 1944), which has resulted in the identification of more than twenty-six free amino acids and related compounds. The subject has been reviewed by Hinton (1954) and Auclair & Dubreuil (1953).

Most of the investigations in the past have concerned the free amino acids in haemolymph of holometabolous insects, and little is known of hemimetabolous forms: 15 amino acids were detected by microbiological assay in *Carausius morosus*, the stick insect (Duchateau, Sarlet & Florkin, 1952), and in *Locusta migratoria* (Duchateau, Florkin & Sarlet, 1952). The same fifteen were found in both species: glutamic acid and histidine proved to be quantitatively important in the stick insect, and glutamic acid and proline in the locust. In both species, the total amount of amino acids in the haemolymph was much less than in most of the holometabolous insects examined (Duchateau, Sarlet & Florkin, 1952; Duchateau, Florkin & Sarlet, 1952; Auclair & Dubreuil, 1952; Chen & Hadorn, 1954). The culicid, *Corethra plumicornis* of the last-named authors, is an exception; its low level of free amino acids (671/μg./100μl.) is comparable with that of *Rhodnius* and *Locusta*.

Haemolymph of insects feeding on blood has also been neglected in studies of free amino acids and other compounds in insects, and this omission was a second reason why the hemipterous, blood-sucking bug was chosen for the present study.

A qualitative and semi-quantitative analysis of the haemolymph was therefore made. This was accompanied by an analysis of free amino acids in the blood of the rabbit which was the laboratory host. A further study was made on the amino acids in *Rhodnius* excreta, and this forms the subject of the second paper of this series.

Throughout this paper, the term 'haemolymph' refers to the circulatory fluid of the insect, and 'blood' to that of the mammalian host.

* Present address: South African Institute for Medical Research, P.O. Box 1038, Johannesburg, South Africa.

MATERIALS AND METHODS

Biological material

Adults and immature stages of *Rhodnius* were used throughout. An account of the biology, rearing and maintenance of *Rhodnius* cultures has been given by Buxton (1930). Cultures were kept at 26° C., and were fed on the ears of a lop-eared rabbit. Haemolymph of adults and 5th- (or final) instar bugs was used predominantly for analyses.

Chromatographic analyses of haemolymph

(*a*) *Withdrawal of haemolymph*

Samples for analysis were withdrawn from living insects. Little difficulty was experienced in collection as the haemolymph does not coagulate. Before withdrawal of samples, the abdomen was washed with distilled water and dried with filter-paper. In the case of adults, the wings were first removed, and an edge of the abdomen was nicked with surgical scissors. The abdomen of the larva was gently pricked with a micro-scalpel or a needle. After gentle manual pressure, the clear droplets of haemolymph which welled from the incisions were collected in graduated capillary micro-pipettes. The haemolymph was transferred to small glass tubes 1 in. to 1½ in. long and ¼ in. diameter where it was treated for chromatographic analysis. Adults yielded 25–30 μl. haemolymph at optimum times after feeding, females providing more than males. About 30–60 μl. could be obtained from 5th-instar nymphs, and 10–15 μl. from 4th instars. For routine studies, 5th-instar haemolymph was generally used because of the greater volume available.

(*b*) *Volumes of haemolymph used*

30 μl. volumes were used for routine analyses. Volumes greater than 50 μl. were usually found to be excessive and often led to the formation of whitish yellow spots due to the separation of inorganic ions of the chromatogram. Such spots may distort the final positions of certain amino acids, e.g. those of aspartic and glutamic acids (Westall, 1948; Dent, 1948), unless the material is first desalted. Larger volumes (50–80 μl.) were occasionally used in attempts to detect ninhydrin-reactive substances which might be present in very low concentration in haemolymph.

(*c*) *Chromatographic procedure*

Proteins were precipitated by the addition of a volume of 80% ethanol about 10 times the volume of the sample, and removed by centrifugation. The clear alcoholic fraction was transferred to a separate tube and the precipitated proteins washed several times with 80% ethanol and each time centrifuged. The total volume of alcoholic solution (including the washings) was evaporated to dryness *in vacuo*, and immediately before analysis taken up in a small volume (20–30 μl.) of 80% ethanol and applied to the paper. Two-dimensional paper chromatography was used almost exclusively; the method adopted was that of Datta, Dent & Harris

(1950), which makes it possible for large numbers of chromatograms to be run simultaneously under identical conditions, and in a shorter time than with larger sheets. With careful technique, separations were obtained which compared favourably with those obtained on larger papers. The method was especially useful in serial studies, such as that of qualitative changes in amino acids after feeding, and before and after ecdysis, and semi-quantitative studies by serial-dilution methods. It also allowed a number of replicates to be run of a single sample, under identical conditions.

Phenol, in the presence of KCN (to prevent oxidation of the solvent) and NH_3 vapour, was used as the first solvent, and a collidine-lutidine mixture in the presence of diethylamine as the second. Both solvent systems were prepared by the method of Auclair & Dubreuil (1952). Papers (Whatman no. 1) were allowed to equilibrate in the respective solvents for 12 hr. before the commencement of a run. At the end of the second development, the frame was finally removed when the yellow solvent front had been drawn to the top of the chromatograms by flat sheets of paper kept in place by thin strips of glass. The dried chromatograms were sprayed with a 0·1% (w/v) solution of ninhydrin in *n*-butanol. Spots were produced by heating the papers to 110° C. in an oven for 3 min.

Identification was made by conventional methods (use of markers, pure substance added to unknown, R_F values in the literature, use of specific reagents, etc.). Acid hydrolysis was carried out on protein-free samples which were heated with 6N-HCl in small sealed tubes for 2–3 hr. at 150° C. (Dent, 1948; Levenbook, 1950). After hydrolysis, the acid was removed by repeated evaporation *in vacuo*, and again after successive additions of small volumes of water. Finally, the sample was made up to a convenient volume (20–50 μl.), and applied to the paper. The isomeric leucines were separated by uni-dimensional chromatography (Work, 1949), after elution of the overlapping spots. Better separations were got after 6 days development instead of the 3 days suggested by Work. Circular paper chromatography (Saifer & Oreskes, 1953) was used for specific testing of certain amino acids and related compounds. The technique was useful for preliminary analyses before more extensive two-dimensional separations.

(d) Semi-quantitative analysis of free amino acids of haemolymph

A serial-dilution method of Auclair & Dubreuil (1952) was used. All determinations were made in duplicate. A series of decreasing volumes of deproteinized haemolymph of 5th-instar bugs was prepared and deposited on separate papers which were then developed. In a second series, decreasing volumes of pure amino acids were applied, and these papers too were developed. Volumes were measured by an Agla Micro-injection Unit capable of delivering 0·2 μl. The papers were sprayed with ninhydrin after development. The concentrations of the particular spots visible for the last time in the series produced under controlled conditions were recorded, and these were compared with the concentrations obtained with the pure standard amino acids in the second series. In this way it was possible to estimate the approximate concentration of each amino acid in any sample of haemolymph.

RESULTS

(*a*) *General characteristics of haemolymph*

The haemolymph of *Rhodnius* is a clear, yellowish fluid of about pH 7·2. It gave positive results for the usual colour tests for proteins, e.g. the Millon, Molisch, xanthoproteic, biuret and ninhydrin reactions. The absence of free tryptophane was demonstrated by consistently negative results after the Hopkins–Cole test. When stood in the air at room temperature, the haemolymph darkened from the exposed surface downwards, due to the formation of melanin. This reaction was prevented when the sample was warmed at 60° C. Complete coagulation occurred at 69–71° C. Darkening was also prevented by the addition of small amounts of KCN, and this inhibition was reversed by addition of Cu^{2+} (as $CuSO_4$) to KCN-treated haemolymph, when characteristic darkening took place. Toluene added to haemolymph also stopped the reaction by preventing the oxygen of the atmosphere from reacting with the haemolymph. The refractive index of the haemolymph is 1·3504 at 24° C., and the specific gravity measured by the falling-drop method of Barbour & Hamilton (1926) had a value of 1·0149 for 3rd-instar insects 2 days after the blood meal. Both the haemolymph volume, measured by the method of Richardson, Burdette & Eagleson (1931), and the specific gravity showed marked increases during the first few days after feeding, suggesting absorption of food material from the gut. From the 7th day, the blood volume remained fairly constant (about 10 μl. for 3rd-instar bugs) until ecdysis. The volume is much affected by the relative humidity of the atmosphere. For example, that of 3rd-instar bugs is maintained constant at 10 μl. for 8 weeks at 90 % R.H., whereas this volume drops to 2·3 μl. at 10 % R.H.

(*b*) *Qualitative analysis of haemolymph*

(i) *The amino acid pattern.* Some eighteen free amino acids and related substances were detected in haemolymph (Table 1).

Amino acids present in all stages of nymphal and adult haemolymph were substantially the same. In a few nymphal analyses, β-alanine was found, but in

Table 1. *Free amino acids and related compounds appearing in the haemolymph of* Rhodnius prolixus

Substance	Intensity*
Valine	Intense
Leucine (as iso-leucine and leucine), glycine, proline, alanine	Strong
Aspartic acid, cystine, cysteic acid, glutamine, histidine, phenylalanine, serine, threonine, taurine	Distinct
Arginine, lysine, tyrosine	Faint
Thyroxine, glutamic acid, asparagine, α-amino-*n*-butyric acid	Faint and rare
β-Alanine, hydroxyproline, methionine, tryptophane, creatine, creatinine, cysteine†	Not detected

* Degree of intensity should not be confused with concentration as ninhydrin reacts differently with different amino acids.

† Cysteine not detected with phenol as solvent (Winegard, Toennies & Block, 1948).

very low concentration. Taurine was found in male and female but not in nymphal haemolymph. The concentration of each amino acid, judged by the overall intensities of the spots, appeared to be about the same in all instars. Several analyses were made of nymphal haemolymph of the related blood-sucking bug, *Triatoma infestans*, and a close resemblance in pattern and concentration to *Rhodnius* haemolymph was seen.

(ii) *Hydrolysis of deproteinized haemolymph.* Hydrolysis showed the absence of conjugated amino acids or peptides. No changes in concentration of most amino acids were produced, except for glutamic acid which increased at the expense of its amide. Aspartic acid showed no noticeable increase, showing that its amide, asparagine, is absent, or present in very low concentration. In one sample of haemolymph a spot agreeing with Dent's (1948) position for α-amino-octanoic acid was recorded. An unidentified amino acid appeared in a single chromatogram, taking up a position directly above leucine.

Peptides were not observed at any time in haemolymph, even in samples up to 10 μl. untreated haemolymph, nor in samples up to 70 μl. of deproteinized haemolymph. Four polypeptides have been reported in haemolymph of *Gastrophilus intestinalis* (Levenbook, 1950), one or more in *Corethra plumicornis*, *Ephestia kuhniella* and *Drosophila melanogaster* (Chen & Hadorn, 1954), and di- and tripeptides in *Oryctes nasicornis* and *Melolontha vulgaris* (Ussing, 1946).

(iii) *Comparison with the amino acid pattern of mammalian blood.* There is little qualitative difference between the *Rhodnius* pattern and that in the blood of the rat, guinea-pig, cat and man (Dubreuil, Auclair & Timiras, 1953). Rabbit serum patterns obtained in the laboratory agree with published evidence. The presence of the unknown compound reported by Dubreuil *et al.* (1953) was confirmed.

Quantitatively, however, there are considerable differences. In *Rhodnius*, histidine, leucine, phenylalanine, proline and tyrosine are at a far higher concentration. To a lesser degree, this applies to valine, cysteic acid and glycine. The remainder are at about the same level. The only plasma amino acids which clearly predominate over *Rhodnius* values are glutamine and alanine.

The total α-amino nitrogen concentration of *Rhodnius* haemolymph determined by the ninhydrin method of van Slyke, MacFadyen & Hamilton (1941) ranges from 46·7 to 111·05 mg. % compared with an average value of 4·1 mg. % in human plasma (Hamilton & van Slyke, 1943), i.e. the α-amino-N content in *Rhodnius* haemolymph is 10–25 times that in human plasma, which is considerably less than that reported for most insects (see Roeder, 1953, p. 172).

(*c*) *Semi-quantitative analysis of haemolymph*

Table 2 shows the values obtained by means of a serial-dilution method. The total amino-N from this table is 69·64 mg. %. Deducting the value for taurine, this becomes 69·48 mg. %. The α-amino-N values obtained for five samples of *Rhodnius* haemolymph by the van Slyke method (van Slyke *et al.* 1941) ranged from 46·7 to 111·05 mg. %. A wide variation is therefore evident among samples taken from

bugs fed at the same time. Nevertheless, all the values are low compared with those found in other haemolymphs where values of the order of 200–300 mg. % are frequently encountered (see Roeder, 1953).

Table 2. *Semi-quantitative analysis of free amino acids in deproteinized haemolymph of 5th-instar* Rhodnius prolixus *withdrawn a week after the blood meal*

Amino acid	Minimum quantity of pure substance (μg.)	Minimum volume of haemolymph showing amino acid (μl.)	Concentration in haemolymph (μg./100/μl.)	Concentration expressed as amino-N* (mg./100 ml.)
Histidine	8·3	3·2	259·4	23·42
Leucine	0·25	0·3	83·3	8·9
Phenylalanine	3·0	4·5	66·6	5·65
Proline	1·5	3·4	44·1	5·37
Tyrosine	1·5	2·6	57·7	4·44
Valine	0·07	0·2	35·0	4·19
Cysteic acid	1·5	3·3	45·5	3·78
Glycine	0·04	0·2	20·0	3·74
Lysine	0·90	2·6	34·6	3·32
Alanine	0·04	0·4	10·0	1·57
Threonine	0·35	3·0	11·7	1·38
Glutamine	0·25	2·6	9·6	0·92
α-Amino-*n*-butyric acid	0·20	3·0	6·6	0·90
Arginine	2·0	20·0	10·0	0·81
Serine	0·1	2·7	3·7	0·49
Aspartic acid	0·15	5·0	3·0	0·32
Glutamic acid	0·1	3·4	2·95	0·28
Taurine	0·3	20·8	1·4	0·46
			705·15	69·64

* Amino-N values from Albritton (1954, p. 102), except α-amino-*n*-butyric acid and cysteic acid, calculated from molecular weights.

(*d*) *Individual amino acids*

Apart from the information supplied in Tables 1 and 2, the following additional data is available. *Histidine*, though appearing as a faint spot with ninhydrin, accounts for about 34 % of the total amino-N. Both it and histamine are quantitatively significant in *Rhodnius* excreta (Harington, 1956). The concentration of *phenylalanine* is also significant; it occurs seldom in the insect haemolymphs studied. Taurine is present in adult bugs but not in immature stages of *Rhodnius*, though it occurs in substantial amount in excreta (Harington, 1956), and is commonly found in mammalian blood.

Thyroxine. This substance, not previously detected in insect haemolymph or tissues, was found in freshly drawn larval haemolymph on four occasions, and in two samples of adult haemolymph which had been allowed to stand for a week during melanin-formation experiments. Its precursors, tyrosine and phenylalanine, are well-established constituents of haemolymph. Identification of the

cases recorded depends upon the use of pure thyroxine as marker, R_F values in the literature (Dent, 1948), and on the fact that thyroxine runs so fast in both the solvents used that it takes up a position well clear of other materials. Because of the rare appearances of thyroxine, it was not possible to confirm its presence further by adding pure substance to the unknown before chromatography. Details of the appearance of thyroxine are given below.

Thyroxine in haemolymph of living insects

(1) In a 3rd-instar larva; in hydrolysate of deproteinized haemolymph. Volume of sample unknown.

(2) In a 4th-instar larva; in hydrolysates of two separate samples of deproteinized haemolymph. Volume: 20 μl. each.

(3) In a 4th-instar larva; in deproteinized unhydrolysed haemolymph. Volume unknown.

(4) In a 5th-instar nymph; in deproteinized, unhydrolysed haemolymph. Volume: 55 μl.

Thyroxine in standing blood

(1) In 40 μl. female haemolymph, stood for 7 days. The black plug at the bottom of the tube was taken up in alcohol, centrifuged, and the clear solution chromatographed. Apart from the appearance of thyroxine, there was an increase in intensity of almost all amino acids over the levels of normal haemolymph. This may be due to protein destruction on standing. Phenylalanine was present in lower concentration though, and tyrosine was absent, both having possibly been utilized during the haemolymph-darkening reaction, or perhaps during thyroxine synthesis.

(2) In female haemolymph, withdrawn 7 days after death. Here again, tyrosine was not detected.

The effect of administered iodine on the presence of thyroxine in Rhodnius *haemolymph*

Experiment 1. Fifth-instar bugs were artificially fed on serum and iodine (2 mg. % serum), and a control group on serum alone. Chromatographic analyses of both groups at intervals up to 6 days showed little variation. No thyroxine was detected.

Experiment 2. In this experiment, potassium iodate was added to a small volume of freshly drawn 5th-instar haemolymph 3 days before ecdysis. The materials were well mixed, and set aside for 3 hr. No distinct darkening was noticed; normal samples darken in 10–30 min. A distinct spot appeared after spraying, in the thyroxine position, and a second, faint grey spot in a position corresponding to di-iodotyrosine.

Mono-iodotyrosine was detected once during routine analyses of normal untreated *Rhodnius*—in 20 μl. of unhydrolysed 3rd-instar blood, and in the absence of di-iodotyrosine or thyroxine.

DISCUSSION

Some twenty-one free amino acids and related compounds have been detected in *Rhodnius* haemolymph by paper chromatography. There is little variation either quantitatively or qualitatively between the amino acid patterns of adults and immature insects. The free amino-N concentration of haemolymph (46·7–111·05 mg. %), though 10–20 times that of human plasma, remains the lowest recorded for insect haemolymph except for values of 40–80 mg. % for *Hydrophilus* (Florkin, 1937) and 94 mg. % for *Gastrophilus* (Levenbook, 1950). Of the total amino acid concentration, 43 % is in the form of basic amino acids (histidine, lysine and arginine), 23 % as monoaminocarboxylic acid (leucine, valine, glycine, threonine and alanine), and 15 % as aromatic amino acids (tyrosine and phenylalanine). The non-protein amino-N of the haemolymph has been found to originate from amino acids only. Amides are present in low concentration (glutamine) or present in traces (asparagine). Peptides were not detected and are probably absent. Histidine was found to be present in highest concentration, accounting for nearly 34 % of the total amino acid concentration. This substance has been detected in high concentration in haemolymph of *Melolontha vulgaris* (Ussing, 1946), in larvae and pupae of certain Lepidoptera (Duchateau, Florkin & Sarlet, 1952; Fukuda & Sakano, 1950; Duchateau & Florkin, 1953), and in *Tenebrio molitor* (Auclair & Dubreuil, 1953).

The detection of thyroxine is probably the first record of this substance in insect haemolymph or tissue. It is unlikely that it was taken into the body via the host blood as thyroxine has not been detected on chromatograms of serum of the rat, guinea pig, rabbit or man (Dubreuil *et al.* 1953), nor in human plasma by Dowex column chromatography (Stein & Moore, 1954), though it is present in small amounts in human plasma (Talbot, Butler, Saltzman & Rodriguez, 1944; Taurog & Chaikoff, 1946), where it is probably in the form of protein-bound iodine (Harington, 1944; Taurog & Chaikoff, 1948).

There is the interesting possibility that it may play an hormonal role in *Rhodnius* metabolism, although its formation in withdrawn haemolymph after addition of iodate may simply be the result of the supposedly non-enzymic formation of the hormone when protein is iodized. Casein and soya bean protein both yield thyroxine equally well on iodination, and apparently the value of the protein for this purpose lies in its content of tyrosine (see Harrow, 1946, p. 480). This amino acid increases in *Rhodnius* haemolymph towards ecdysis and, in the single experiment performed, it would be available for thyroxine formation. Its disappearance from the haemolymph after iodination further suggests that the formation proceeded as described (see Roche & Michel, 1951). The thyroxine which appeared in the haemolymph of living insects might have been formed in the same manner, assuming that sufficient iodine and tyrosine were available. It remains to be seen what would happen to *Rhodnius* during ecdysis if iodate were to be administered while tyrosine is freely available for thyroxine formation.

SUMMARY

1. A qualitative and semi-quantitative analysis of the free amino acids in the haemolymph of *Rhodnius prolixus* has been made by paper chromatography.

2. Twenty-one amino acids or related compounds were detected of which histidine appears at the highest concentration.

3. The α-amino-N concentration of haemolymph is among the lowest values recorded for insect haemolymphs.

4. A substance corresponding in position to thyroxine has been detected in haemolymph of a few immature insects, and is formed if iodate is added to freshly withdrawn haemolymph.

I wish to record my great appreciation to the late Professor P. A. Buxton, C.M.G., F.R.S., for his valuable assistance and criticism during this study and to members of the staff for helpful discussions. The study forms part of a Thesis for the degree of Ph.D. approved by the University of London, and was financed by the South African Council for Scientific and Industrial Research whose generous support I acknowledge with pleasure.

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Studies of the amino acids of *Rhodnius prolixus*

II. Analysis of the excretory material

By J. S. HARINGTON

*Department of Entomology, London School of Hygiene and Tropical Medicine**

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Studies of insect excreta are long overdue, and even since the advent of such techniques as partition chromatography, they have been greatly neglected in favour of studies of haemolymph, a surprising fact when one considers that it is only a study of both which can provide the beginnings of some concept of intermediary metabolism in the organism. What we know of the subject has been reviewed by Craig (1960).

Thus the present study complements a detailed qualitative and semi-quantitative chromatographic analysis of the free amino acids of *Rhodnius* haemolymph (Harington, 1961*a*).

MATERIALS AND METHODS

Biological material

The excretory products of *Rhodnius* can be divided into three groups:

(*a*) The urine, a clear, copious, colourless fluid passed soon after the blood meal.

(*b*) A yellow pultaceous mass drying as a powder, called here 'the yellow fraction'.

(*c*) 'The black fraction'—black masses of excreta representing the residue of the previous meal (Wigglesworth, 1931).

As will be described in the text, (*b*) and (*c*) are of identical amino acid composition and the black fraction differs only in that it is contaminated with haematin which masks the yellow colour of the excretory pigment.

Chromatographic analyses of excretory material

Collection of material. Adult urine was collected directly after the blood meal from waxed watch glasses by Wigglesworth's method (1931), and the volumes were recorded in graduated micro-pipettes. No deproteinization was necessary, and samples were transferred directly to the paper chromatograms. Desalting, too, was unnecessary as at the volumes used, no great distortion of amino acid positions was caused by salt spots, and also because few amino acids appeared in the urine anyway.

Urine was analysed before and after hydrolysis with 6N-HCl at 150° C. for 3 hr. Qualitative analyses were made of over fifty specimens of urine, and a semi-

* Present address: South African Institute for Medical Research, P.O. Box 1038, Johannesburg, South Africa.

quantitative estimation made by a serial dilution method (Auclair & Dubreuil, 1952). Yellow and black fractions of excreta were treated identically. No attempt was made to analyse excreta at successive time intervals after feeding. Samples were collected from the filter paper in the jars containing 5th-instar and adult bugs, and transferred to small glass tubes where they were ground up after the addition of a small volume of 80% ethanol. Samples were pooled and ranged in weight from 25 to 100 mg. After the addition of a larger volume of ethanol to deproteinize the samples, they were allowed to stand for 10 min., after which they were centrifuged and repeatedly washed with additions of ethanol. The alcoholic solution was evaporated to dryness *in vacuo*, and immediately before chromatography taken up in a small volume of ethanol and applied to the paper.

Excreta prepared by the more elaborate method of Awapara (1948), and used by Yoshitake & Aruga (1950*a*) for analysis of silkworm faeces did not give improved results when compared with the alcoholic treatment followed in the laboratory. Samples of yellow, black and mixed fractions were analysed before and after hydrolysis, and chromatographic development, spraying and identification were carried out as described in paper I of this series (Harington, 1961*a*).

At a late stage in this investigation it proved possible to analyse excreta of *Rhodnius* and of the related blood-sucking bug *Triatoma infestans* by the column separation method of Moore, Spackman & Stein (1958), using a fraction column 20 cm. long with IR 120 ion exchange resin in the sodium form. The results obtained are compared with those obtained by paper separations.

RESULTS

Qualitative analysis of excretory material

(*a*) *The urine*

Studies on the volume of urine excreted after the blood meal agree with those of Wigglesworth (1931). Females excreted much more urine than males, owing to their greater weight and greater blood meals. *Rhodnius* urine has been examined in detail by Wigglesworth (1931) and, as Patton (in Roeder, 1953) points out, this represents the only extensive analysis of insect urine.

Almost all the nitrogen excreted by *Rhodnius* is in the form of uric acid (Wigglesworth, 1931); the present study shows that amino acid excretion is low, but that quite significant amounts of certain amino acids appear in the yellow and black fractions. The urine pattern is unchanged after hydrolysis; in fact, no peptides or conjugated amino acids were detected at any time.

Individual amino acids. Cysteic acid appears consistently in urine and to a lesser degree *cystine*. The latter was detected without previous oxidation with H_2O_2 (Dent, 1948). The most common urinary pattern was simply a single spot representing cysteic acid, with cystine frequently appearing and a yellow inorganic spot due to the excretion of unwanted salts in the diet.

A semi-quantitative estimation of the two sulphur-containing amino acids was carried out by the method of Auclair & Dubreuil (1952). Serial dilutions of urine and of standard solutions of pure substance gave the following results (Table 1).

Cysteic acid is therefore present in urine at about the same concentration as in

haemolymph (45·5 μg./100 μl.), and since it was not obtained by oxidation of cystine on the paper, both substances are presumably excreted in their present form. Identical amino acid patterns were obtained from urines of bugs fed on serum only.

Table 1. *Approximate concentrations of cysteic acid and cystine in urine of* Rhodnius prolixus

Amino acid	Minimum quantity detected (μg.)	Minimum volume of urine giving spot (μl.)	Concentration in urine (μg./100 μl.)
Cysteic acid	1·5*	3	50
Cystine	0·2*	15	1·3

* Cysteic acid value from Harington (1961*a*); in the absence of a value for cystine, that of Auclair & Dubreuil (1952) was used.

In a few other cases, amino acids other than these appeared in urine. In several samples of about 30 μl. in volume, *phenylalanine* appeared for the first time, but was not always present in high volumes of urine. In one case, in 50 μl. adult urine, collected 30 min. after the end of the blood meal, the following amino acids appeared: *tryptophane*, *leucine*, *valine*, *alanine*, *glutamine*, *arginine*, *glutamic acid*, *cysteic acid*, and three spots in the area of the latter substance and which might be its decomposition products. This sudden appearance of a spate of amino acids in the usually barren urine is probably the first flushing-out effect of the early urination after the blood meal.

(*b*) *The yellow and black fractions*

A preliminary note on the amino acid pattern of this material, especially relating to histidine and histamine, has already been published (Harington, 1956). The results obtained after analysis of the yellow fraction are shown in Table 2, together with the results of an analysis performed on a Moore and Stein column (Moore, Spackman & Stein, 1958), using 150 mg. dry excreta of *Rhodnius*.

No differences, except for minor qualitative variations, were found in the amino acid patterns of yellow, black and mixed fractions. Samples of excreta collected from the related blood-sucking bug, *Triatoma infestans*, showed no qualitative difference on paper, and on the column were quantitatively very similar to the *Rhodnius* pattern except for a slightly higher urea figure (3·2 c/f 2·3 %) which is hardly significant. It should be remembered that the percentages ranging from 0·003 to 0·007 in Table 2 represent trace amounts present. Ammonia is present in high concentration, and there is an intense basic undetermined substance running ahead of lysine.

Individual amino acids. Leucine appeared in fairly high concentrations and was regularly detected. *Taurine* appeared in about 25 % of the chromatograms run, and when present was found in substantial amount. It is commonly found in normal and pathological urines of man (Dent, 1948). *Phenylalanine*, *arginine* and

lysine appeared occasionally, *alanine* and *glutamic acid* more frequently. Cystine was found in a third of the chromatograms run and in small amounts.

Hydrolysis of excreta showed no change in amino acid pattern except the disappearance of two of the yellow pigment spots.

Table 2. *Paper and column chromatographic analysis of the yellow excretory fraction of* Rhodnius prolixus

Amino acid or related substance	Column separation (results expressed as percentage of 150 mg. dry excreta analysed)	Paper separation (intensities given)
Yellow pigment	Not determined	+ + + +
Histidine	Not determined; very intense	+ +
Histamine	Not determined	+ +
Lysine	0·036	−
Leucines	0·019	+ + +
Valine	0·019	+
Glutamic acid	0·015	−
Taurine	0·013	+
Phenylalanine	0·007	+
Threonine	0·005	−
α-alanine	0·004	+
Methionine	0·004	−
Arginine	0·003	−
Glycine	0·003	+
Urea	2·3	−
Ammonia	Not determined; intense	−

The yellow pigments. Careful chromatographic analysis by a number of different solvents of substantial amounts of dry excreta (100–250 mg.) indicated the presence of three separate yellow pigment spots on the chromatograms. All three are referred to as pigments since they could be seen as distinct yellow spots on the dry chromatogram without any prior treatment with staining reagents. In unidimensional chromatograms with *n*-butanol–acetic acid–H_2O (40:10:50, v/v; Partridge, 1948), three yellow spots could be seen on the dried strips. Their characteristics are shown below (Fig. 1).

The reactions of spots B and C to ninhydrin and acid hydrolysis (when new ninhydrin-positive spots are formed) indicates that both are peptides. Spot C is quantitatively by far the most important pigment spot, and a detailed study of its chemical constitution will be reported in detail elsewhere. It is not the yellow pigment of the wings of the butterfly, *Gonepteryx rhamnii*, described by Hopkins in 1896, as at one time suggested (Wigglesworth, 1931); the butterfly pigment is a mixture of leucopterins and xanthopterins (Good & Johnson, 1949), all fluorescent but ninhydrin-negative, and with chromatographic characteristics quite unlike the *Rhodnius* pigments as was shown by comparative studies here. Preliminary evidence is in favour of spot C being a tripeptide (cystine, cysteine, glutamic acid?), possibly related in terms of R_F values to glutathione (glutamylcysteinylglycine) in

butanol–acetic acid–H_2O as solvent. In addition to this, the strong fluorescent property of spot C confers upon it a particular interest, especially if it is a peptide.

Other substances in excreta. Almost all the nitrogen in *Rhodnius* excretion has been reported to occur in the form of *uric acid* of which 80–90 % exists in the free form (Wigglesworth, 1931). This substance was identified on chromatograms in the present study by means of a $AgNO_3$–NH_3 spray followed by heating at 100° C. for 3 min. (Dent, 1947). R_F values were 0·19 (phenol) and 0·45 (collidine). Five other substances appeared after the use of this spray, and represent *reducing substances* in the excreta. It was out of the scope of the present work to attempt their identification. Urea was detected in high concentration by column chromatography.

Tests for hexosamines and *N*-acetylhexosamines (Partridge, 1948) gave negative results, as did Sakaguchi and Jaffé tests for guanidines and creatine and creatinine respectively. Creatine has, however, been reported present, and to increase during successive days after feeding (Wigglesworth, 1931).

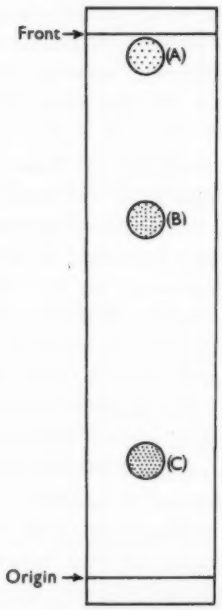

Fig. 1. Typical one-dimensional chromatogram, run by ascent overnight at 25° C. in butanol–acetic acid–H_2O. Inspected before and after treatment with ninhydrin and ultra-violet examination. Other ninhydrin-reactive spots are omitted for clarity. Spot A: R_F 0·98 (mean of three runs); pale yellow fluorescence, ninhydrin-negative, survives acid hydrolysis. Very often absent, unless large amounts of excreta are used (200–250 mg.). Spot B: R_F 0·61 (mean of 13 runs); sky-blue fluorescence, ninhydrin-positive, disappears on acid hydrolysis. Regularly present in excreta above 70–80 mg. weight. Spot C: R_F 0·18 (mean of 9 runs); bright yellow-green fluorescence, ninhydrin-positive, disappears on acid hydrolysis. The major pigment spot, regularly present in small amounts of excreta (10–20 mg.) and upwards.

DISCUSSION

The value of studies on the free amino acids of excreta will increase when more information is available for comparative purposes. For example, a few limited but quantitative studies on *Triatoma* excreta have shown that it is for all purposes almost identical to that of *Rhodnius*, which is what might have been expected. Brown (1937) found arginine present, creatine and creatinine absent, and cystine doubtful in the excreta of the grasshopper, *Melanoplus bivittatus*, and Wigglesworth's detailed analysis (1931) of *Rhodnius* urine has been discussed frequently in this paper. As far as free amino acids and related substances are concerned, the urine of *Rhodnius* after the blood meal consists only of cysteic acid, cystine and inorganic salts, and the significance of these has already been discussed elsewhere (Harington, 1961*b*). In the later excreta (the yellow and black fractions), seven amino acids were found—leucine, glycine, valine, alanine, phenylalanine, taurine and histidine, together with histamine and three yellow pigment spots, one of which is ninhydrin-negative. All of these substances, except histamine and the pigments, have been detected in silkworm faeces by Yoshitake & Aruga (1950*a*) who in addition found glutamic acid, glutamine, aspartic acid, tyrosine, and very small amounts of serine and threonine. To this must be added Kawase's early finding (1915) of proline and uric acid.

The high concentration of histidine in both haemolymph and excreta of *Rhodnius* is similar to that found in the silkworm. Here histidine (and leucine) increase in amount in the faeces during growth and development and reach maximal concentrations in the final instar (Yoshitake & Aruga, 1950*a*). In addition, the urine of adult silkworms contains a large amount of histidine, especially just after pupation (Yoshitake & Aruga, 1950*b*). Histidine occurs in larval haemolymph of the silkworm (Fukuda, 1950) and in high concentration in pupal haemolymph (Fukuda & Sakano, 1950) where it increases in concentration during pupation to reach its maximum just before the emergence of the adult when the level again falls (Yoshitake & Aruga, 1950*b*). A relationship between histidine in haemolymph and histidine and histamine in excreta of *Rhodnius* has been proposed by Harington (1956). The quantitative importance of histidine in both *Rhodnius* and the silkworm (whose diets are greatly different) suggests that this substance might be of particular physiological importance to both insects, although at the same time there might be sufficient histidine in mulberry leaves to possibly account for high levels in haemolymph and excreta of the silkworm. Microbiological assay of mulberry leaf hydrolysates gives a figure for histidine of 0·46% dry weight (Fukuda, 1960; personal communication.)

Of the four amino acids present in greatest concentration in *Rhodnius* haemolymph—histidine, leucine, phenylalanine and tyrosine—the first three are present in excreta, histidine and leucine in high concentration. Phenylalanine is not strong and tyrosine absent, both of these are substrates in great demand for ecdysis and are probably utilized then (Fukuda, 1956).

It is probable that some form of regulatory mechanism (osmotic?) controls the level of haemolymph amino acids within a fairly constant range for it has been

shown that if large quantities of an amino acid are ingested, part appears immediately in the haemolymph, part in the early urine and the remainder in the later excretory fractions (Harington, 1961*b*).

Beadle & Shaw (1950) found that there was a regulation of the non-protein N in the haemolymph of *Sialis lutaria* to compensate for fluctuations in the chloride concentration. It is thought that this regulation is concerned with the equilibrium between the haemolymph proteins and the haemolymph amino acids, though nothing is known of the stimulus which would bring about increased or decreased hydrolysis.

Finally, mention should be made of the finding of high concentrations of urea and ammonia in both *Rhodnius* and *Triatoma* excreta, especially since ammonia does not occur in *Rhodnius* urine (Wigglesworth, 1931) and it is not common in the excreta of terrestrial animals generally.

SUMMARY

1. A qualitative paper chromatographic analysis of the free amino acids in *Rhodnius* urine and excreta was made: eleven amino acids or related compounds were detected, cystine and cysteic acid in urine, and histidine, histamine, leucine, taurine, glycine, valine, phenylalanine and alanine in the excretory fractions.

2. In addition to these, three yellow pigment spots were separated from the excreta; one of these is of unknown composition and two are peptides.

3. A possible relationship between certain of the excretory amino acids and haemolymph ones is discussed.

I am greatly indebted to the late Professor P. A. Buxton, C.M.G., F.R.S., for his stimulating criticism during this study, and to the members of the School for very useful discussion, and to Mr Paul Baily for performing the column chromatographic analyses on my behalf. The study forms part of a Thesis for the degree of Ph.D. approved by the University of London, and was financed by the South African Council for Scientific and Industrial Research. I wish to acknowledge their generous financial support.

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The *in vitro* action of immune pig serum on second- and third-stage *Ascaris suum* larvae

By L. F. TAFFS, Ph.D., M.R.C.V.S.

Department of Animal Pathology, School of Veterinary Medicine, Madingley Road, Cambridge

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INTRODUCTION

One of the most interesting earlier observations on immune phenomena in parasitic infections was described by Blacklock, Gordon & Fine (1930), who were the first to demonstrate the formation of precipitates around a metazoan parasite *in vitro*. They showed that when larvae of the fly *Cordylobia anthropophaga* were immersed in homologous immune guinea-pig serum, precipitates invariably formed around them, whereas no precipitate was observed when such larvae were placed in non-immune serum.

Sarles (1937, 1938) showed that a similar reaction occurred when larvae of *Nippostrongylus muris*, a nematode parasite of rats, were placed in homologous immune rat serum. Sarles noticed that precipitates appeared at the natural orifices of the larvae, namely the mouth, the excretory pore, the anus, and also round the cuticle and in the intestine. Earlier, Sarles & Taliaferro (1936) had shown that rats infected with *N. muris* develop an acquired immunity. They thought resistance had to a large extent an antibody basis, because (1) it was passively transferable, and (2) it might involve a precipitin, as precipitates were found around the anterior end and in the intestine of worms retained in the skin and lungs of immune rats. Sarles (1937, 1938) and Taliaferro & Sarles (1939) believed that their results provided direct evidence for the action of antibody on the parasite, and that precipitates, which were seen both in the immune host and *in vitro*, formed as a result of an affinity between antigen and antibody.

Since the classical studies of Sarles, this *in vitro* reaction has been observed in other parasitic conditions such as infestations with hookworms (Otto, 1939), *Trichinella spiralis* (Mauss, 1940) and schistosomes (Vogel & Minning, 1949).

In infections with *Ascaris suum*, Oliver-Gonzalez (1943) and Soulsby (1957) have demonstrated, in rabbits and guinea-pigs, respectively, a similar *in vitro* reaction. However, as far as the writer is aware, precipitates have not yet been shown with immune serum of the pig, the natural host of the parasite. The aim, therefore, of this investigation, was first, to find out if such a reaction occurred when *Ascaris* larvae were immersed in immune pig serum of proven antibody content; and secondly, to ascertain if there was any relationship between the antibody titre of the serum and the amount and type of circumlarval precipitate (oral, cuticular, excretory and anal) which formed following immersion of the larvae in antisera of varying antibody content. It was thought that, if precipitates

were observed, and if a correlation were found between the antibody titre and the amount of precipitation, this would confirm that sera which were positive according to the conglutinating complement absorption test did contain antibody, which was also present in amounts proportional to the titre of the serum.

Two experiments are described on the action of immune pig serum on: (1) third-stage *Ascaris* larvae, and (2) second-stage *Ascaris* larvae.

MATERIALS AND METHODS

Third-stage Ascaris *larvae*

These were obtained by killing two heavily infected guinea-pigs on the seventh day of infection and recovering the larvae from the lungs by means of the standard Baermann technique. After sedimentation of the larvae by centrifugation, they were passed through cotton-wool in a funnel containing water at 37° C. The cotton-wool filter removed extraneous matter and the larvae were collected beneath the funnel in a clean condition. The larvae were then washed in distilled water, placed for 15 min. in 0·1 % mercuric chloride solution and were then rinsed repeatedly in sterile saline. This procedure removed many bacteria and hence helped the preparations to remain in good condition for a longer period of time (Sarles, 1938).

Second-stage Ascaris *larvae*

These were obtained by the artificial hatching of embryonated eggs in which the larvae had undergone the first moult. To facilitate hatching, the outer albuminous coat of the eggs was removed by incubating the eggs overnight at 37° C. in 90 % 'Milton'* (containing 1 % sodium hypochlorite and 16·5 % sodium chloride). The eggs were then concentrated by centrifugation and washed repeatedly in saline. A number of these eggs were placed on a glass slide and a coverslip placed over them. By repeated tapping of the coverslip the walls of the eggs were broken down and the majority of the second-stage larvae were freed. They were then washed off the slide, passed through a cotton-wool filter and were treated with 0·1 % mercuric chloride as previously described.

Pig sera

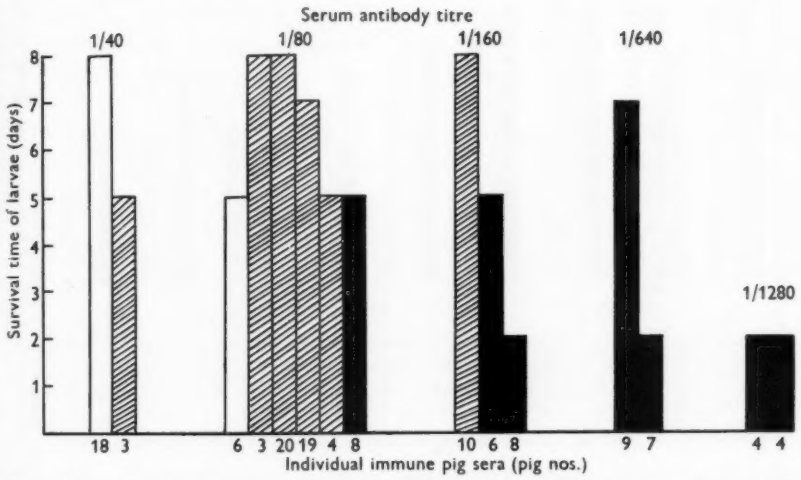
Both third- and second-stage larvae were immersed in fourteen different samples of non-immune sera (negative), fifteen different samples of immune sera (positive) and in normal saline. The sera had been collected from six control and ten pigs experimentally infected in earlier experiments.

All the sera had been previously tested for antibody against *Ascaris* by means of the conglutinating complement absorption test, using a saline extract of whole worm as antigen. No fixation of complement had been observed in any of the negative pig sera, and the positive immune sera showed titres ranging from 1/40 to 1/1280. All the sera were inactivated at 56° C. for 30 min. and were used undiluted.

* Milton Antiseptic Ltd.

The in vitro *preparation*

The method used was similar to that described by Sarles (1938). Two small concentric rings of autoclaved 'Vaseline'* were made on a sterile slide. A drop of the test serum or saline and five or six *Ascaris* larvae were placed within the smaller ring. A sterile coverslip was then pressed down carefully so as not to restrict the movement of the larvae. The slides were kept at room temperature and were examined at least three times a day for 12 days. Notes were made at each observation concerning the activity of the larvae, the degree of precipitation around the larvae, the type of precipitate which formed, and the period for which the larvae survived. The criterion of death of the larvae was the absence of movement following repeated warming of the slide.



Text-fig. 1. Survival time of third-stage larvae of *Ascaris suum*, and estimated amount of circumlarval precipitate at time of death, in fifteen samples of undiluted immune pig serum grouped according to the antibody titre-conglutinating complement absorption test. Estimated total precipitate in preparation at death of larvae: □ slight; ▨ medium; ■ heavy.

RESULTS

A. *Experiment* 1. *Action of immune pig serum on third-stage larvae*

The *in vitro* effect of normal and immune serum, and saline on third-stage larvae is summarized in Table 1. No precipitate formed around any larvae which were placed in saline or normal serum. Circumlarval precipitates, which were first seen in some slides 3 hr. after immersion of the larvae, were observed in all preparations containing immune serum of titres ranging from 1/40 to 1/1280. Precipitates formed around the mouth, excretory pore, anus and the cuticle of the larvae (Pl. 1, figs. 1–6). A greater quantity of precipitate was seen around larvae which were placed in sera of high titre (Text-fig. 1), and the higher the titre of the serum, the sooner precipitates began to form (Table 1). The excretory precipitate

* 'Vaseline' Brand Petroleum Jelly, Chesebrough–Pond's Ltd.

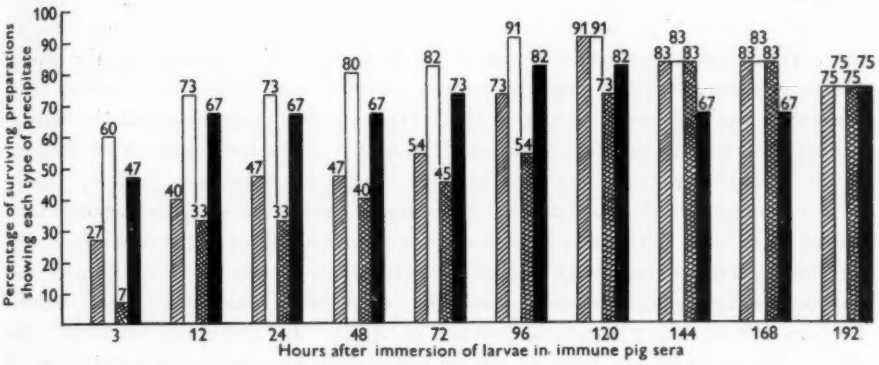
Table 1. *The* in vitro *effect of normal and immune pig serum on third-stage larvae of* A. suum

Pig no.	Serum antibody titre (C.C.A.T.)	Survival time larvae‡ (days)	Degree of precipitation at death	Types of precipitate observed at different intervals of time after immersion of larvae in serum																															
				3 hr.				12 hr.				24 hr.				48 hr.				72 hr.				96 hr.				120 hr.				144 hr.				168 hr.				192 hr.			
				O	E	A	C	O	E	A	C	O	E	A	C	O	E	A	C	O	E	A	C	O	E	A	C	O	E	A	C	O	E	A	C	O	E	A	C	O	E	A	C
Normal serum																																									
Saline	0	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.	.	.	.
11	0	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16	0	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16	0	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.	.	.	.
16	0	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16	0	12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.	.	.	.
17	0	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.	.	.	.
17	0	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	0	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	0	10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19	0	9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.	.	.	.	.
19	0	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.	.	.	.	.
20	0	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.	.	.	.	.
20	0	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	.	.	.	.	.
20	0	7	–																																					
		Av. 7·5 (Not saline)																																						
Immune serum																																								
3	1/40	5	••	–	–	–	–	–	+	–	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.
18	1/40	8	•	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–	–	–
3	1/80	8	••	–	–	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–	+	–	–	–	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	1/80	5	••	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	+	+	–	+	+	+	–	+	.	.	.	.	.	.	.	.	.	.	.
6	1/80	5	•	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.
8	1/80	5	•••	–	+	–	–	+	+	–	+	+	+	–	+	+	+	–	+	+	+	–	+	+	+	–	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.
19	1/80	7	••	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	+	+	–	–	+	+	+	–	+	+	+	–	.	.	.
20	1/80	8	••	–	+	–	+	–	+	–	+	–	+	–	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	1/160	5	•••	–	+	–	+	–	+	–	+	–	+	–	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.
8	1/160	2	•••	+	+	–	+	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10	1/160	8	••	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	+	+	–	+	+	+	–	+	+	+	–	+	+	+	–	+	+	+	–	+	+	+
7	1/640	2	•••	–	+	–	+	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9	1/640	7	•••	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.
4	1/1280	2	•••	+	+	–	–	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	1/1280	2	•••	+	+	+	+	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		Av. 5·2																																							
Total positive				4	9	1	7	6	11	5	10	7	11	5	10	7	12	6	10	6	9	5	8	8	10	6	9	10	10	8	9	5	5	5	4	5	5	5	4	3	3	3	3
Percentage positive				27	60	7	47	40	73	33	67	47	73	33	67	47	80	40	67	54	82	45	73	73	91	54	82	91	91	73	82	83	83	83	67	83	83	83	67	75	75	75	75

O = Oral precipitate. E = Excretory pore precipitate. A = Anal precipitate. C = Cuticular precipitate.
+ = Presence of precipitate in the preparation. – = Absence of precipitate in the preparation. * = Estimated total precipitate at death of larvae. C.C.A.T. = Conglutinating complement absorption test.
N.B. (i) Each preparation (slide) contains five or six larvae in serum at room temperature. (ii) ‡ '*Survival time larvae (days)*'—denotes time at which *all* the larvae are found dead.

Percentage of surviving preparations showing each type of precipitate

often appeared first and was followed by the cuticular, oral and anal precipitates (Text-fig. 2). For the first 96 hr. of immersion of the larvae in the immune sera, more slides showed excretory and cuticular precipitates; thereafter oral and anal precipitates were just as frequently seen (Text-fig. 2). Larvae lived longer in normal sera (average survival time 7·5 days) than in immune sera (average survival time 5·2 days), and in immune sera of low titre they survived for a longer period of time than when they were immersed in immune sera of high titre (Text-fig. 1). Larvae were more active in normal sera than in immune sera, and were less active in antisera of high antibody content, as compared with antisera of low antibody content.



Text-fig. 2. The rate at which oral, excretory pore, anal and cuticular precipitates formed around third-stage larvae of *Ascaris suum* immersed in fifteen samples of immune pig serum. ▨ oral; □ excretory; ▩ anal; ■ cuticular.

B. *Experiment* 2. *Action of immune pig serum on second-stage larvae*

When second-stage larvae were immersed in samples of the same immune serum, no precipitates formed around any larvae; neither was any difference noted in the life span of the larvae nor in their activity when they were placed in saline, normal or immune sera.

DISCUSSION

The results show that precipitates develop at the mouth, excretory pore, anus and on the cuticle of third-stage larvae after their immersion in immune pig serum and that complement is not necessary for this reaction to take place.

Precipitate was most evident at the excretory pore and was often the first precipitate to appear. Sarles (1938) also recorded the early appearance and gradual increase in amount of the excretory precipitate in his *in vitro* studies on *Nippostrongylus muris*. Further, Soulsby (1957) noted that precipitate was most evident at the excretory pore of *Ascaris* larvae which had been placed in homologous immune guinea-pig serum. He suggested that the materials emanating from this opening represented the major part of the antigenic material available. From these observations it would appear that the excretory products play an important part in stimulating the production of antibody in the host.

* = Estimated total precipitate at death of larvae. C.C.A.T. = Conglutinating complement absorption test.
N.B. (i) Each preparation (slide) contains five or six larvae in serum at room temperature. (ii) ‡ 'Survival time larvae (days)'—denotes time at which *all* the larvae are found dead.

The amount of precipitate which formed around the larvae, the period for which the larvae survived, the activity of the larvae, and the rate at which precipitates formed were correlated with the antibody titre of the serum. A similar result was obtained by Oliver-Gonzalez (1943), who used the interfacial precipitin test in rabbits infected with *A. suum*. He showed that when *Ascaris* larvae were immersed in immune rabbit serum at varying intervals after infection, the percentage of larvae showing oral precipitate bore a direct relationship to the precipitin titre of the serum; thus when the titre rose so did the percentage of larvae showing oral precipitate.

The present results have shown that pig sera, which were positive according to the conglutinating complement absorption test, did contain antibody, which was also present in amounts proportional to the titre of the serum. Moreover, since circumlarval precipitates form *in vivo* in guinea-pigs (Soulsby, 1957), and possibly also in pigs (Taffs, 1958) resistant to *A. suum*, and that a correlation can be demonstrated between resistance and high serum antibody titres (Taffs, 1958), it appears that the titre of the serum is in some degree a measure of the ability of the host to resist reinfection.

No precipitates were observed around second-stage larvae. Soulsby (1960) also was unable to demonstrate the presence of precipitates around second-stage larvae after their immersion in homologous immune guinea-pig serum. In newly hatched young second-stage *Ascaris* larvae, little structural development has taken place and larval metabolic products are probably small in amount. Indeed, Soulsby (1958), using a natural marker antibody, the Forssman antibody, has shown that it is at the time of the second moult, i.e. about the fourth day of infection, that antigen release can first be detected in any large amount. The necessary prerequisite for the formation of precipitate is that there must be sufficient antigenic material present to react with specific antibody, so that the absence of precipitate may be due to the lack of antigen.

Immune guinea-pigs show little resistance before the fourth or fifth day of reinfection. The absence of precipitates around second-stage larvae *in vitro* may partly explain this lack of resistance shown by the host to young second-stage larvae in the early migratory phase, in that only small amounts of antigenic material liberated from the larvae would be available to react with, or stimulate the production of, antibody in the host. A rise in serum antibody occurs about 4 or 5 days after reinfection (Taffs, 1958), and Soulsby (1957) has seen precipitates around larvae attempting to migrate in immune guinea-pigs about this time. It would seem, therefore, that by the fourth and fifth day of reinfection, sufficient antigenic material would have been released from the larvae to stimulate the production of more antibody, which would then, in combination with antibody already present in the serum, react with the larval antigens to the ultimate detriment of the migrant larvae.

SUMMARY

Third and artificially hatched second-stage larvae of *A. suum* were exposed to the action of saline and normal and homologous immune pig serum. Precipitates developed at the mouth, excretory pore, anus and around the cuticle of third-stage

larvae when they were placed in immune pig serum, but not in saline or normal serum. Precipitate was most evident at the excretory pore and was often the first precipitate to form. It is suggested that the excretory products may play an important role in stimulating the production of antibody in the host. A relationship was noted between the antibody titre of the serum and the amount of precipitate, the life span of the larvae, their activity, and the rate at which precipitates formed. It is concluded that the titre of the serum was in some degree a measure of the ability of the host to resist reinfection.

Circumlarval precipitates did not form when second-stage larvae were placed in samples of the same immune serum. It is pointed out that this may be due to the lack of sufficient antigenic material available to react with antibody in the antisera. It is also suggested that an absence of sufficient larval antigen may also partly account for the small degree of resistance exhibited by the resistant host within the first 2 or 3 days of reinfection.

The work, which is abstracted from a Ph.D. thesis, was carried out in 1957 under the tenure of an Animal Health Trust Wellcome Fellowship. I am grateful to Dr Soulsby, under whose guidance this work was carried out.

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EXPLANATION OF PLATE 1

Plate 1 shows the *in vitro* appearance of circumlarval precipitates after the immersion of third-stage *A. suum* larvae in immune pig serum.

Figs. 1, 2 and 4 × 416; Fig. 3 × 333; Figs. 5 and 6 × 124.

Figs. 1–3. Anterior ends of larvae showing precipitate at the mouth and excretory pore.

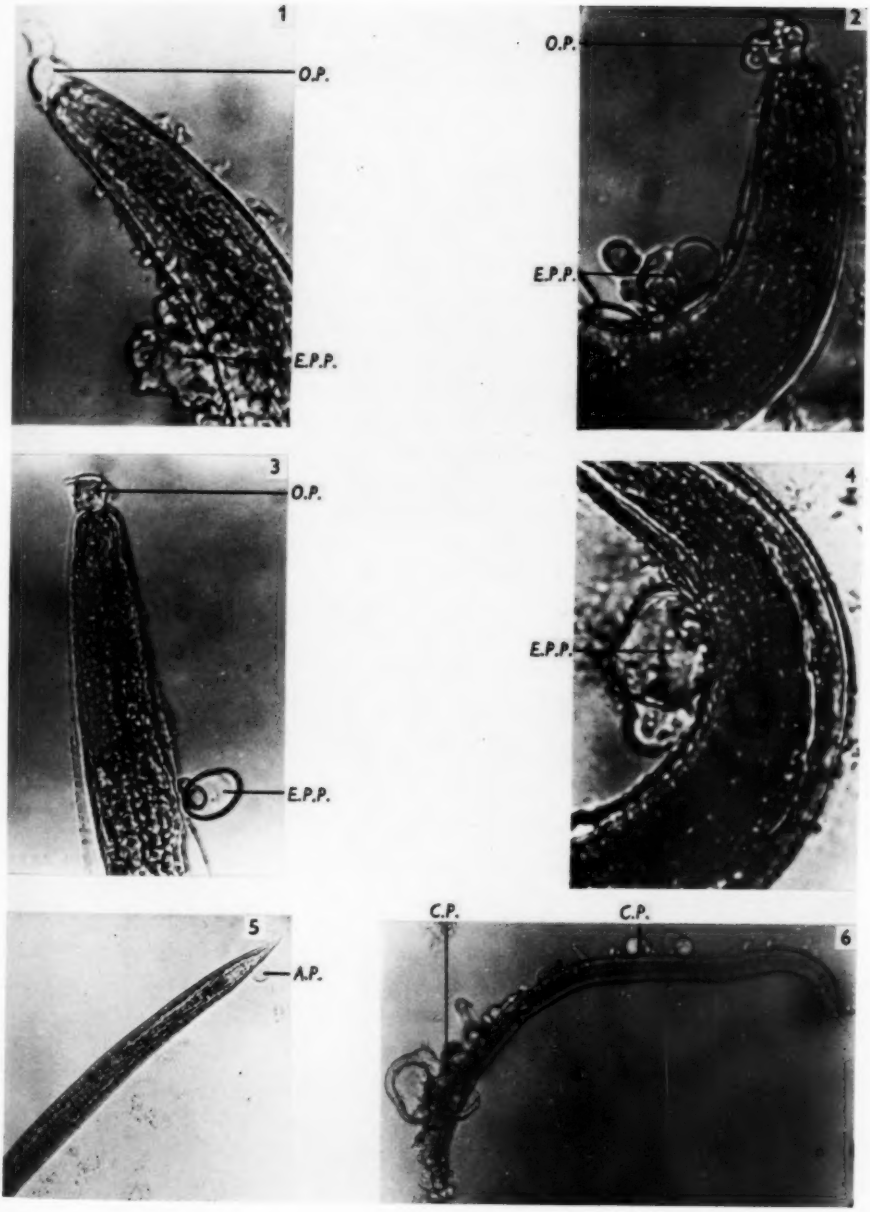
Fig. 4. Precipitate at the excretory pore of a larva.

Fig. 5. Posterior end of a larva showing precipitate at the anus.

Fig. 6. Larva showing precipitate on the cuticle.

KEY TO LETTERING OF FIGURES

O.P. = Oral precipitate
E.P.P. = Excretory pore precipitate
A.P. = Anal precipitate
C.P. = Cuticular precipitate



L. F. TAFFS

(*Facing p.* 334)

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Physiological studies on trematodes—*Fasciola gigantica* carbohydrate metabolism

By MADAN M. GOIL

Department of Zoology, Bareilly College, Bareilly, U.P., India

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INTRODUCTION

Physiological studies on some species of trematodes have been reported by Goil (1957, 1958*a–c*, 1959). The present paper is a continuation of these studies. The trematode *Fasciola gigantica*, which is very common in India, was selected for them. No data about it are available, although extensive studies of *F. hepatica* have been made. A further aim of the work was to find out whether the specificity of the hosts plays any important part in the metabolic behaviour of the parasites. Earlier workers have collected *F. hepatica* from the sheep, goat and cow, whereas in the present studies *F. gigantica* was collected from buffaloes.

MATERIAL

The parasite was collected from the bile ducts of buffaloes slaughtered at the local abattoir.

METHODS

The flukes obtained were washed in normal saline till they were free of debris, and were then washed several times in sterile saline. They were then blotted quickly on filter-paper and weighed. The dry weight was determined by drying to a constant weight at 100° C.

The glycogen was estimated by the method of Seifter, Daytons, Novie & Muntwyler (1950). For *in vitro* experiments for analysing the consumption of glycogen the method suggested by Rogers (1952) was used. The parasites were kept starved for a period of 10 hr. and the glycogen consumption was determined. The medium in which the parasites were kept was quantitatively analysed in each instance after 2 hr. of incubation for lactic acid by the method of Barker & Summerson (1941).

RESULTS

(*a*) *Total glycogen content before starvation.* The results of fifteen analyses carried out with several lots of parasite showed that the glycogen content ranged from 3·7 to 6·3 % with an average of 5·4 % ± 0·16 of fresh weight (F.W.) and from 17·39 to 29·61 % with an average of 25·35 % ± 0·78 of dry weight (D.W.) of tissue.

(*b*) *Total glycogen content after starvation period of* 10 *hr.* The results of fifteen analyses carried out showed that the glycogen content in the parasite ranged from 2·8 to 4·6 % with an average of 3·5 % ± 0·85 of F.W. and from 13·16 to 19·74 % with an average of 16·29 % ± 0·58 of D.W. of tissue.

The statement (Table 1) shows the glycogen content before and after starvation.

(*c*) *Analysis of the medium for lactic acid.* The lactic acid given out by the parasite after 2 hr. of incubation ranged from 0·17 to 0·93 % with an average of 0·46 % ± 0·25 of F.W. and from 0·8 to 4·37 % with an average of 2·18 % ± 0·38 of D.W. of tissue.

Table 1

Total glycogen content of parasite in g./100 g. of F.W. Mean of 15 observations and range	Total glycogen content of parasite after starving for 10 hr. in g./100 g. of F.W. Mean of 15 observations and range	Quantity of glycogen consumed after 10 hr. of starvation in g./100 g. of F.W.	Glycogen consumed in 10 hr. as % of total glycogen content
5·4 ± 0·16 (3·7 to 6·3)	3·5 ± 0·85 (2·8 to 4·6)	1·9	35·2

DISCUSSION

The commonest carbohydrate reserve in these endoparasites is glycogen and they derive from this most of their energy in an anaerobic environment. The previous data on the glycogen content of the trematodes of the bile ducts were obtained by studies of *F. hepatica* (Flury & Leeb, 1926; Weinland & von Brand, 1926) and *Paramphistomum explanatum* (Goil, 1957). In *F. hepatica* the glycogen content recorded by Flury & Leeb was 3·1 % of F.W. or 15 % of D.W. of tissue, while Weinland & von Brand recorded 3·7 % of F.W. or 21 % of D.W. of tissue. In *P. explanatum* it was 7·26 % of F.W. or 30·32 % of D.W. of tissue. In *F. gigantica* it was found to be 5·4 % of F.W. or 25·35 % of D.W. of tissue. It is difficult to assign any definite reasons for the higher percentage of glycogen in *F. gigantica* as compared to *F. hepatica*. It is interesting, however, to note that a fairly large quantity of stored glycogen was recorded by the author in *P. explanatum*, which was also collected from the bile ducts of buffaloes. It may be that the different feeds given to the host in the different countries may play an important role in the metabolism of the parasites which they harbour. Another probable reason could be that the parasites may be storing up their carbohydrate reserve for periodical emergencies, such as malnutrition of their hosts, so that the parasites may tide over this lean period.

The information available about the rate of consumption of carbohydrates by the trematodes of the bile ducts seems to be very meagre. The rate of consumption by *F. gigantica*, which was found to be 35·2 % in 10 hr., agrees very well with the rate of consumption of glycogen (36·4 %) recorded in *P. explanatum* by Goil (1957). The rate of glycogen consumption by *F. hepatica* recorded by Weinland & von Brand (1926) was determined under anaerobic conditions and was 2·6 % g. of F.W. of glycogen in 24 hr. The data on the glycogen consumption in the different parasites recorded by von Brand (1950) show that anaerobic or aerobic conditions do not make a remarkable difference in glycogen consumption, but in the present work the aerobic rate of glycogen consumption by *F. gigantica* was found to be much higher than the anaerobic rate of *F. hepatica*. It is just possible that the energy release in the two species of *Fasciola* may be different.

Quantitative figures for the lactic acid production of trematodes kept under anaerobic conditions are available for *Schistosoma mansoni* (Bueding, 1950), in which species it accounts for 80% of the utilized carbohydrate. In *F. hepatica*, the lactic acid has not been recorded as an end product, though it is possible that it has not been investigated. In *F. gigantica* the aerobic lactic acid production is 0·46% of F.W. within a period of 2 hr. But other end products were not studied in the present investigations, so that nothing definite about the quantitative aspect of these in relation to glycogen content can be said.

SUMMARY

1. Studies have been made of carbohydrate metabolism in *Fasciola gigantica* during starvation and these are compared with data available about carbohydrate metabolism in *F. hepatica*.

2. In *F. gigantica* the glycogen content before starvation was 5·4% of F.W. or 25·35% of D.W. of tissue, and the glycogen content after starvation was 3·5% of F.W. or 16·29% of D.W. of tissue.

3. The medium in which *F. gigantica* was incubated was analysed for lactic acid as one of the end products of glycogen consumption. The lactic acid given out in a period of 2 hr. was 0·46% of F.W. or 2·18% of D.W. of tissue.

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An *in vivo* screening method for anthelmintic activity using *Hymenolepis nana* var *fraterna* in mice

By J. CROWLEY, B.Sc.

Veterinary Science Division, Boots Pure Drug Co. Ltd, Thurgarton, England

INTRODUCTION

Tapeworm infestations are still common in man and domestic animals; in particular *Echinococcus granulosus*, with its threat to man in the form of hydatid cysts, is still widespread, and there is, as yet, no efficient anthelmintic with a wide spectrum of activity.

Of the taenicides and taenifuges in general use many were discovered empirically, many are natural products and thus tend to vary from batch to batch and the activity of some is in doubt. The action of some drugs is specific, but a remedy with a wide spectrum of activity is preferable, so that it would seem that a reliable screening method by which a large range of compounds could be tested for activity against tapeworms is desirable. *Hymenolepis nana* in mice has been used for this purpose by Steward (1955) and Cavier (1956) and *H. diminuta* in rats by Seelkopf (1952), and dogs have been used by several workers (Bondareva, 1954; Craige & Kleckner, 1946; Enzie, 1944; and Stewart, 1937), but primary testing in large animals is expensive and time consuming, so that drugs cannot be covered in large numbers. *In vitro* tests have been used by Duguid & Heathcote (1950) employing *Moniezia* on a kymograph and by Vanni (1953) employing the oligochaete *Limnodrilus*, but they give only a rough guide to activity, because very often a drug given to an animal is altered by digestion before it can reach the worms and in addition *in vitro* testing will indicate drugs that are toxic to all living organisms. The use of *H. nana* in mice as a basis for screening overcomes these difficulties and provides a reliable guide to those drugs which are worth testing in larger animals.

CHOICE OF HOST AND INFESTATION

Hymenolepis nana was chosen as a suitable test infestation for screening for the following reasons: it has an armed scolex; it is easily obtained and can be used in its natural host to which it is well adapted; the intermediate host is not obligatory; and, if eggs are used to produce infestation, the risk of large secondary burdens is minimal. The definitive host is normally maintained in most laboratories, is cheap to breed, easy to handle and, being small, needs only a small amount of drug. *H. nana* is also a parasite of man.

H. nana is preferable to *H. diminuta*, because the latter needs an intermediate host, and, unlike most of the *Taenia* group, has an unarmed scolex and there might be difficulty in establishing an infestation in mice from a stock which also carried *H. nana*, because according to Heyneman (1953), *H. nana* protects mice against *H. diminuta* by cross-immunization.

Mice infested by means of cysticercoids acquire tapeworms which are situated high in the small intestine and, because there is no tissue stage involved, the host does not become immune. Under these conditions, eggs from the primary infestation can invade the mucosa successfully, the resulting cysticercoids confer immunity, and a large secondary burden of worms, which may number thousands of dwarf individuals, is established in the lower small intestine. Such infestations tend to lose the primary burden of one or more large worms and are unsuitable for drug testing, because they do not readily respond to anthelmintics and the variation in the numbers of worms established is so great that comparison with controls becomes unsatisfactory. If eggs are used, large secondary burdens are uncommon in healthy mice which are not carrying any great numbers of other parasites, but, when they occur, the tests should be repeated.

MAINTAINING THE INFESTATION

Because young mice are more susceptible, 3-week-old weaner mice are used for infesting, but, because these are small and have a short intestine, the eggs tend to be swept through before they have time to invade the intestinal villi. To overcome this difficulty, morphine hydrochloride is given with the eggs to slow down peristalsis (Larsh, 1947).

The majority of breeding stocks of mice carry a natural infestation with *H. nana* and, because pregnant mice are twice as susceptible as controls to infestation (Larsh, 1949), and also because larvae derived from eggs produce a strong immunity which can be passed to the young through the placenta and in the milk (Larsh, 1942), better infestations are obtained if the breeding stock is kept clean. Also a previous infestation in the weaner mice is undesirable, because a strong immunity is established within as little as 12 hr. after the primary infestation and lasts about 23 weeks. Even in the absence of the worm, immunity lasts 20 weeks (Hearin, 1941).

Elimination of infestation in the stock mice may be effected by fumigating the breeding room to kill the mites and insect intermediate hosts which may carry infestation and by frequent cleaning of cages and dosing of the adult stock with a taenicide. Fumigation is carried out monthly with 3 g. of γ-BHC per 1000 cu.ft. vapourized for 4 hr., using an electric fumigator. A suitable taenicide is 1-diethyl aminopent-4-yldithiocarbamic acid, which has been used by the author at a rate of 0·25 mg./g. In weaners from untreated breeding stock artificial infestations show a 6 weeks to 2 months cycle.

METHOD

The strain of *H. nana* used was obtained from naturally infested mice bred at Thurgarton.

Fresh gravid *H. nana* are ground in a mortar, water is added and the suspension is passed through a 90-mesh per inch sieve into a 2 in. Petri dish to a depth of approximately $\frac{1}{2}$ cm. This culture is allowed to stand at room temperature for 3 days and then the number of infective eggs present in 0·15 ml. (measured with a McDonald pipette) is counted and an estimation is made of the total number

present. Infective eggs are refractile and contain a hexacanth embryo and the culture is diluted so that 0·1 ml. contains 200 infective eggs. Three-week-old weaners weighing between 10 and 15 g. are dosed with 0·1 ml. of the egg suspension mixed immediately before use with equal parts of a fresh 0·05% solution of morphine hydrochloride. Dosing is done with a 1 c.c. graduated tuberculin syringe attached by a short length of soft rubber tubing to an open-ended 1-in. length of gum elastic No. 2 catheter.

Fourteen days after infestation one mouse per test drug is dosed at 1 mg./g. body weight in the first instance, unless toxicity data indicate that a lower dose is preferable. In order to give an accurate dose, the drugs are ground with dry gum arabic and then water is added to give a 5% solution or suspension of the drug and this suspension is given with the syringe and catheter at the rate of 0·4 ml. for a 20 g. mouse, 0·44 ml. for a 22 g. mouse, etc. Liquids are emulsified in 10% gum arabic solution. Care should be taken that the catheter is well down the oesophagus and that none of the dosing material comes back into the mouth. The dosed mice are caged separately in wire-mesh-bottomed cages, the droppings are collected daily for 3 successive days, soaked in water overnight, washed with a 60 mesh per inch sieve and searched for *Hymenolepis*. The pieces of worm are recorded as gravid or mature, it being impossible to distinguish between mature and immature pieces without staining, and pieces over 1½ cm. are recorded as 'long'. Unless the mouse is negative at autopsy the passage of gravid pieces of worm is of no significance if they are less than 1½ cm. long, because this happens in the normal life history of the parasite. Unfortunately the scolex is very rarely recovered.

After 7 days the mice are weighed, killed and left over night in the refrigerator before autopsy, to allow the worms to relax and become detached. A comparison of dosing and killing weights can be used as some evidence of toxity, bearing in mind that control mice caged individually are inclined to lose a little weight.

The pieces collected from the droppings serve to indicate activity and to distinguish between those mice which have passed all their worms and those which were uninfested at dosing.

Initially the high dose level of 1 mg./g. is given and if the drug does not show 100% activity it can be discarded. If activity is shown, or if the mouse dies within 7 days, the drug is repeated at a lower dose rate. When the approximate limit of good activity is found the drug is administered to four mice at the same dose level and the average worm count on autopsy is compared with the average in four controls. An efficiency of 40% or less is not considered to be of any significance. If, on the other hand this test is promising, a much larger batch of mice is dosed and compared with the same number of controls. Two confirmatory tests were performed in order to conserve mice, because, when good activity of the drug has been recorded in one mouse, it is often not possible to repeat it on a larger scale.

Controls pass gravid pieces of worm 3–4 mm. long and occasionally mature pieces of similar length; but the passage of pieces longer than 1½ cm. has been observed only on extremely rare occasions.

Some drugs, for example, dichlorophen, allow the worms to be digested or destroyed by bacterial activity *in situ* and it may be thought that a mouse dosed with an active drug of this type would be mistaken for one which was uninfested at dosing. In practice, although the worms may be very degenerate, it is almost always possible to detect them by laboratory examination and in any event this possibility can be covered by repetition of the test.

RESULTS WITH KNOWN ANTHELMINTICS

It is considered that a screening test, to be reliable, should show activity with known remedies given in a single dose and the following table shows the results obtained by dosing mice with some taenicides and taenifuges in general use.

Table 1. *Known anthelmintics dosed to mice*

	Drug	Dose (mg./g.)	Mice died / Total dosed	Efficiency (%)	Remarks
1.	Mepacrine	0·15	0/4	50	—
		0·2	1/4	100	—
		0·25	1/4	100	—
2.	Dibutyl tin dilaurate	0·25	0/6	40	—
		0·5	4/5	94	—
		1	2/2	100	—
3.	Lead arsenate	0·5	0/4	50	—
		1	0/8	100	—
4.	Kamala	0·5	0/8	72	—
		1	2/4	100	—
5.	Dichlorophen	0·25	0/5	78	—
		0·5	0/18	89	—
6.	Extract of 24–26% B.P. *Filix mas*	0·4	0/4	40	—
		0·6	0/4	38	—
		0·8	0/10	59	—
7.	Copper sulphate	0·2	1/7	48	2 mice were cleared
8.	Copper + nicotine sulphates	0·15 / 0·000025	1/4	47	—
9.	Arecoline hydrobromide	0·0005	0/5	0	—
		0·001	0/7	0	—
		0·002 up to 0·2	0/16	0	1 mouse per dose rate
		0·3	1/1	—	—
10.	Arecoline Acetarsol (as Nemural*)	0·5	0/4	65	—
		1	0/4	55	—

* Bayer Products Ltd.

(1) Mepacrine is a compound which has been widely used in the treatment of malaria in both man and animals and it has been found to have taenicidal properties in addition. It is used in the treatment of *Taenia* spp. in man, for which it is effective, and for *Hymenolepis nana* in man at a dose of 0·8–1 g. in divided doses,

though opinions as to the efficiency for *H. nana* vary from poor to very good. It is quite effective against *Taenia* spp. and *Dipylidium* spp. in dogs if it is given at the rate of 20–40 mg./kg., but poor for *Echinococcus* spp. even at 50 mg./kg. Against *H. nana* in mice mepacrine was 100 % efficient at doses of 0·2 mg./g. and over, though rather toxic.

(2) Dibutyl tin dilaurate is the best drug for use against poultry cestodes at the rate of 75 mg./kg. and is very active against *H. nana* in mice, but only at a dose rate which proved to be toxic.

(3) Lead arsenate is commonly used for the treatment of *Moniezia* spp. in ruminants, sheep being given a dose of 0·5–1 g. and cattle 1–2 g., and this dose rate is effective for *Moniezia* spp., but has no action against *Thysanosoma*. A dose rate of 1 mg./g. is not toxic and 100 % active against *H. nana* in mice, but the activity falls off sharply with lower doses.

(4) Kamala, like filix mas is a natural product. It is good for *Dibothriocephalus latus* in dogs at the rate of 2–8 g. and may be used in cats at 1–3 g. and poultry at 0·5–2 g. but its efficiency in the last two hosts is low. Against *H. nana* in mice it is quite efficient at 0·5 mg./g. and 100 % efficient though toxic at 1 mg./g.

(5) Dichlorophen has proved to be of use against *Taenia* spp. and *Dipylidium* spp. in dogs at 200 mg./kg., but its action against *Echinococcus* spp. is poor. It can be used for cats at 100–200 mg./kg. and has some action against *Moniezia* spp. and *Thysanosoma* in sheep at 10 g. It has little action against the poultry cestodes. Against *H. nana* in mice it is active and not toxic at 0·25 and 0·5 mg./g. Tapeworms killed by this drug tend to be digested and destroyed before being passed and are hard to find in the faeces.

(6) Extract of filix mas is one of the most efficient anthelmintics for *Taenia* spp. in man and has some action on *Dibothricephalus latus* and *Hymenolepis nana* in man. It is given in doses of 3–6 mg. of 24–26 % extract with some care, because this is quite close to the toxic level. Filix mas has been superseded for dogs, because its action on the *Taenia* spp. of this host and *Echinococcus* spp. is poor, though it has some effect on *Mesocestoides* spp. It is unsafe for cats, but it can be used for sheep at 4–6 ml. and for cattle at 15–45 ml. Against *H. nana* in mice its efficiency is low. This is a toxic drug which is not easily used because of its viscosity.

(7, 8) Copper sulphate is recommended for *Moniezia* in sheep at the rate of 0·25–1 g. and for cattle at 1–5 g., but its low standard of efficiency is slightly increased if given to sheep as 20–30 ml. of a solution containing 5 % of 40 % nicotine sulphate solution and 5 % of copper sulphate. It has no effect on *Thysanosoma actinioides*. Against *H. nana* in mice the efficiency of copper sulphate is low, being only 48 % at a dose of 0·2 mg./g. (although two of the seven mice dosed were cleared of tapeworms), and approximately the same when mixed with nicotine sulphate. The latter was so toxic to mice that it could not be tested with any degree of accuracy.

(9) Arecoline hydrobromide is dangerous to cats but is used for dogs at a dose rate of 1–1·5 mg./kg., in spite of its toxic effects, particularly when the dogs are infested with *Echinococcus*. Batham (1946) is of the opinion that *Taenia* spp., *Multiceps* spp. and *Dipylidium* spp. are removed as well as *Echinococcus* to the extent of 90–100 % when a fairly high dose rate is used. Arecoline is fairly efficient

for *Dibothriocephalus latus*, but poor in its action on *Mesocestoides* spp., even at doses of 4–15 mg./kg. Mice dosed with arecoline hydrobromide show the griping typical of this drug, but this seldom results in expulsion of Hymenolepis.

(10) Arecoline acetarsol is a rather less toxic drug which can be recommended for cats at 9–18 mg. and poultry at 4·5–9 mg. as well as for dogs at 18–72 mg. and is reported as being efficient. Against *H. nana* in mice it has only a moderate, though repeatable, activity.

It will be seen that of the drugs most commonly considered to be the best tapeworm remedies, arecoline, dibutyl tin dilaurate, dichlorophen, filix mas, lead arsenate and mepacrine, only arecoline and filix mas do not show good results when dosed to mice, although filix mas shows efficiency when it is given in a series of doses, and arecoline is a taenifuge with few taenicidal properties which partly depends for its action on stimulation of the vagus nerve of the host and subsequent purging, an effect which does not seem to result in the passage of *H. nana* from mice.

Though the known anthelmintics are nearly all effective against *Hymenolepis nana*, a much larger dose per gramme is needed than that usually recommended for larger domestic animals and this should be borne in mind when screening new compounds. Also mice will tolerate a dose level which is likely to prove toxic to larger animals.

DISCUSSION

Three other screening tests using *Hymenolepis* have been published, that of Seelkopf (1952), which employs *H. diminuta* in rats, and those of Steward (1955) and Cavier (1956), which both used *H. nana* in mice. Seelkopf's method was used in the testing of male fern extract and phloroglucinol derivatives and the preparations were administered by duodenal sound 3 hr. before the hosts were destroyed. Steward's test was designed for general use and requires ten mice per dose rate, ten undosed controls, and ten positive controls dosed with tetrachlorethylene as a primary test. The mice are fasted before dosing, purged twice with magnesium sulphate after dosing, and fasted before being killed on the third day after treatment. Cavier's test also requires starving and purging and the mice are killed on the day after dosing.

In the test described in this paper the number of mice required has been drastically cut down as compared with Steward's figures, this being made possible by the treatment of breeding stock, which results in a more even infestation of weaners and by searching the droppings for tapeworm segments, which provide proof of infestation in mice found to be negative at autopsy. Also it is considered undesirable to starve the host, because tapeworms are sensitive to changes of the intestinal environment and the infection may be partially or wholly lost by this procedure.

The test described above has proved satisfactory in the routine screening of drugs for anthelmintic activity and large numbers of compounds can be covered, using few mice because the technique incorporates as much as possible of the principle of Hall's critical test (1921). Nearly all the known taenicides tested by this method are active.

SUMMARY

An *in vivo* test is described for the screening of compounds for anthelmintic activity using *Hymenolepsis nana* var *fraterna* in mice. Results obtained with known taenicides and taenifuges are tabled, analysed and compared with their action against the tapeworms of larger animals including man.

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Studies on the resistance of rats to the nematode *Nippostrongylus muris* (Yokogawa, 1920)

I. Dosage–mortality relationship

By G. C. HUNTER and L. C. LEIGH
The Rowett Research Institute, Bucksburn, Aberdeen

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For testing the resistance of the rat to *Nippostrongylus muris* (Yokogawa, 1920), Africa (1931) and Schwartz, Alicata & Lucker (1931) used two methods which have been adopted by subsequent workers. In the first method, counts are made of the number of worms in the rats at autopsy and compared with suitable controls. In the second method worm egg counts are used for comparison. Worm counts 10 days after infection are, in our experience, the most consistent and reliable method, but it involves much time and labour. Another method of comparing the resistance of animals, used by pharmacologists and others, is to give the animals in the treated and control groups a lethal dose or a 50% lethal dose (LD50) and compare the number of survivors and non-survivors in the two groups; statistical analysis being carried out according to Finney (1952). This method, when it is used for rats infected with *N. muris*, will obviously require less work. Perhaps it may not be so sensitive as worm counts, yet there are many experiments in which the information required does not warrant the amount of work involved in counting worms at autopsy. Before this method could be used, it was necessary to investigate the dosage–mortality relationships in normal rats and to estimate the LD50. This has been done by exposing rats to different levels of infection as described in the two series of trials below.

MATERIALS AND METHODS

Rats. Lister hooded rats of heterozygous stock, not previously infected with *N. muris*, were used. The weights of the males ranged from 230 to 370 g., a few weighing up to 450 g. The females weighed from 170 to 300 g.

Nippostrongylus muris. The larvae used were from a stock of *N. muris* maintained for many generations in similar hooded rats as described by Leigh (1956).

Estimation of the number of larvae. The larvae were collected from Baermann funnels and put in a 50 ml. measuring cylinder (A_1), concentrating them if necessary. They were thoroughly mixed by means of a plunger and, before they had time to sink, a sample was taken off with a mercury pipette (see below) and placed in another 50 ml. measuring cylinder (B). The volume in cylinder B was made up to 50 ml. with distilled water. Then the suspension in cylinder B was thoroughly mixed and a sample taken with an ordinary pipette with a fairly wide nozzle was placed in a Peter's eelworm counting slide. The number of larvae in 1·0 ml. was

counted, and the number of larvae taken from cylinder A_1 in the mercury pipette was calculated. The volume in cylinder A_1 was adjusted to give the number of larvae in the mercury pipette required to produce the highest level of infection (i.e. 20,000 larvae/0·25 ml. in the first series). Aliquots from cylinder A_1 were transferred to cylinders A_2, A_3, ..., A_6 (one for each infection level). Cylinders A_2 to A_6 were diluted with sufficient distilled water to give the right number of larvae in the mercury pipette for each infection level. When the desired concentrations had been obtained in the cylinders, six counts (as for A_1 above) were done on each for an estimate of error.

When larvae are handled in water, they sink fairly quickly. In order to get representative samples for counting or infecting, the larvae must be in suspension randomly distributed through the liquid, and the sample containing the required number of larvae in suspension must be taken up quickly. A throttled pipette, which quickly takes up the required volume of liquid and from which the whole amount may be expelled at once, meets these needs. Leigh (1956) described a pipette for this purpose, which was throttled with mercury. A drop of clean mercury is placed in a glass capillary tube about 6 in. long. Both ends of the tube are plugged with cotton wool. At one end there is a rubber teat and at the other a glass nozzle to hold the sample is attached with rubber tubing. The distance the mercury can travel between the cotton wool plugs regulates the volume of liquid which will be taken up in the nozzle of the pipette. The pipette is adjusted to hold 0·25 ml. and is calibrated by weighing a series of samples of distilled water. The coefficient of variation of the one used here was of the order of 1% and the 95% confidence limits of the true mean volume could be set at approximately $\pm 0{\cdot}7\%$ on each side of an observed mean of ten samples.

If the same pipette is used for estimating the number of larvae and infecting the rats, then the volume of the pipette need not be known accurately. It is sufficient to know how many larvae are in a sample taken by the pipette. The use of different pipettes introduces another source of error.

Infection of rats. *N. muris* larvae are skin penetrators and the rats were infected in the following manner. The rats were anaesthetized with veterinary 'Nembutal'.* 1 ml. 'Nembutal' was diluted with 4·5 ml. sterile distilled water and given intraperitoneally at the rate of 0·055 ml./25 g. body weight. The abdomen of the anaesthetized rat was washed with soap and warm water and well rinsed to remove the soap. Surplus water was removed with a piece of absorbent tissue. This removes grease and provides a wet surface on which the drop containing the larvae spreads out and remains on the abdomen; a larval suspension rolls off an unwashed rat. The rats were infected by placing a suspension of larvae on the wet area of the abdomen and returned to their individual cages to dry and recover from the anaesthetic.

First series of trials

In the first series of trials there were six levels of infection; the rats receiving doses of 8000, 10,000, 12,000, 16,000, 18,000 or 20,000 larvae. Groups of six rats,

* Pentobarbitone sodium B.P. 60 mg./ml. Abbot Laboratories Ltd.

either all male or all female, were allocated so that one rat in each group received one of the six doses. After infection the rats were examined every morning and the number of deaths and survivals was noted. Records were also kept of the number of days from infection to death, of weights and of litter numbers.

Results

Table 1, which gives the mortality at the different dosage levels, suggested that females were more susceptible than males and that there were possibly differences in susceptibility from one litter to another. Live weight did not seem to have any influence on survival or death. A probit analysis of the relation between percentage mortality and log dose (or dosage), showed that the results obtained were consistent with the assumption that the lethal dosage value for individual rats was normally distributed, and confirmed that the mean lethal dosage was significantly higher for males than for females ($P < 0{\cdot}05$). The LD50 for male rats was 13,200 (95% confidence limits 11,200–15,600) and for females 9800 (95% confidence limits 8200–11,600).

Table 1. *Mortality at different dosage levels* (*series* 1)

	Male rats		Female rats	
Dose	Number	Deaths	Number	Deaths
8000	6	0	9	4
10,000	7	1	9	3
12,000	7	4	9	7
16,000	7	5	9	8
18,000	7	7	9	8
20,000	7	5	9	9

Table 2. *Infection levels in second series of trials*

Eestimated mortality (from series 1) ...	10%	30%	65%	85%
Dose (No. of larvae) Males	8000	11,000	15,000	20,000
Dose (No. of larvae) Females	6000	8000	11,000	15,000

In the above results a ratio of 1·35 between the two LD50's, based on 41 male and 54 female rats respectively, is significant only at the 5% level. This emphasizes the fact that, when only death or survival is noted, the amount of information per rat is small compared with, for example, the more tedious method of counting the worms at autopsy. In order to obtain more experience of the method and more information on its precision, a second series of trials was carried out which differed from the first series in that:

(1) Each group of rats was from one litter and of one sex. This meant that fewer levels of infection may be tested at the same time.

(2) Different doses were given to males and females, to aim at equal mortalities (see Table 2). When additional rats of the same sex and litter were available, the

range of doses was extended. A fifth male rat was given 6000 larvae and a sixth one got 28,000 larvae, while the fifth and sixth female rats were given 20,000 and 5000 larvae respectively.

Results *Second series of trials*

Table 3 gives the mortality at the different levels of infection for the second series of trials. These results were similar to the first series, so the two sets of data were amalgamated for combined analysis, which showed that:

(*a*) The results were again consistent with the hypothesis that the lethal infection level for individual rats is normally distributed.

(*b*) The LD50 was higher for males than for females ($P < 0{\cdot}05$).

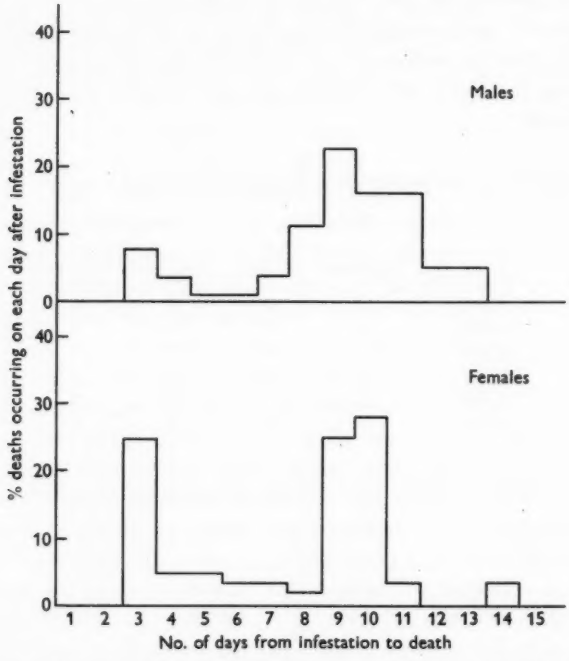

Fig. 1. Distribution of times of death of male and female rats after infestation with *Nippostrongylus muris*.

Table 3. *Mortality at different dosage levels (series 2)*

Male rats			Female rats		
Dose	Number	Deaths	Dose	Number	Deaths
6,000	6	0	5,000	7	1
8,000	11	2	6,000	9	1
11,000	11	5	8,000	9	2
15,000	11	8	11,000	9	6
20,000	11	10	15,000	9	8
28,000	6	6	20,000	9	8

(*c*) Within neither sex was there any evidence of a relationship between lethal dose and weight, and so it is unlikely that the sex difference in susceptibility is purely a function of weight difference.

(*d*) The distribution of times of death was clearly bimodal with peaks at 3 days and between 9 and 10 days after infection (see Fig. 1). The larvae are in the lung at 3 days; thereafter they pass on to the small intestine via the bronchi, trachea and oesophagus. Nine to ten days is the time when the greatest number of mature worms are present in the small intestine. Thus there appear to be two causes of death corresponding to different stages in the life cycle of the worm. As the dose was increased, a greater proportion of deaths occurred at the earlier (lung) stage, but the position of the two modes did not change. Despite the complication of two causes of death, a satisfactory probit regression line was obtained linking dosage and percentage mortality, which makes it seem likely that rats dying at the lung stage would not in any case have survived the gut stage.

The following more accurate values of LD50, based on tests of 97 male and 106 female rats, can therefore be considered as applying either to mortality at the gut stage or to overall mortality:

Males LD50 = 12,300 (95% confidence limits 10,900–13,800).

Females LD50 = 9700 (95% confidence limits 8600–11,000).

It would be difficult to obtain an LD50 applying to early deaths alone because of possible border line cases and also because higher doses would be required.

SUMMARY

The LD50 of *Nippostrongylus muris* for outbred hooded rats has been estimated. It was higher for males than for females and this does not appear to be due to weight differences. The distribution of times of death was bimodal; the peaks corresponding to different stages in the life cycle of the worm.

We wish to thank Mrs M. Whitehead for technical assistance and Mr I. McDonald for statistical analysis.

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Studies on the resistance of rats to the nematode *Nippostrongylus muris* (Yokogawa, 1920)

II. Different routes of immunization and test infection

By G. C. HUNTER and L. C. LEIGH
The Rowett Research Institute, Bucksburn, Aberdeen

(*Received* 7 *November* 1960)

Yokogawa (1922) observed that the parasitic nematode of the rat, *Nippostrongylus muris* (Yokogawa, 1920), entered its host by the skin. Africa (1931) and Schwartz, Alicata & Lucker (1931) first showed that rats which had been infected with the live larvae of *N. muris*, developed an immunity to subsequent infection. This actively acquired immunity has been confirmed by many authors since. While hyperimmunizing rats by placing increasing doses of larvae, at fortnightly intervals, on the skin of the abdomen, Taliaferro & Sarles (1939) found that the skin eventually showed an 'intense residual reaction'. Our own experiments with hooded rats immunized with four large weekly doses of larvae confirmed this skin reaction and we found subsequent necrosis where the larvae had been placed. We also observed that the skin reaction was not confined only to the site of immunization; for example a test dose on the skin of the shoulder would elicit a response on the shoulder of a rat which had been immunized by placing larvae on the abdomen.

When this stage of sensitivity is reached the rats are highly resistant to the parasite and, if resistance and skin sensitivity are correlated, the skin reaction would be a valuable test for studying the immune rat. At present the only satisfactory test of immunity is by direct infection and comparison with non-immune controls. Two experiments were therefore designed to provide some information about the skin reaction and immunity.

PLAN OF EXPERIMENTS

Although the normal route of infection is through the skin, *N. muris* will complete its life cycle if given orally or intraperitoneally. Use was therefore made of the fact that the skin could be avoided and different routes of immunization and test infection were compared. In the first experiment the rats were immunized by giving them live larvae by routes other than the skin, and their resistance was tested by a lethal dose of larvae placed on the skin. In the second experiment the rats were immunized by the usual method of placing the larvae on the skin and the lethal challenge dose was given by other routes.

Design of Experiment I

Different routes of immunization. Treatment groups

I. Non-immune controls.
II. Immunizing infections of larvae given by the mouth.
III. Immunizing infections of larvae given intraperitoneally.
IV. Immunizing infections of larvae given through the skin.

The immunizing infections of larvae were approximately 2150, 4375, 6125 and 7950 larvae given at 7-day intervals. The test infection was 20,000 larvae given 1 week after the last immunizing infection and all the rats received it on the skin. This dose was shown by Hunter & Leigh (1961) to be lethal for approximately 80% of normal hooded rats receiving it on the skin.

Design of Experiment II

Different routes of test infection. Treatment groups.

I. Non-immune controls. Test infection intraperitoneal.
II. Skin immunization. Test infection by the mouth.
III. Skin immunization. Test infection given intraperitoneally.
IV. Skin immunization. Test infection through the skin.

Rats used for Expt. II were immunized with the same numbers of larvae at the same intervals as those used for Expt. I. The test infection of 20,000 larvae was given 1 week after the last immunizing infection by the route stated for each group.

MATERIALS AND METHODS

Outbred male hooded rats, between 3 and 3½ months old and weighing 280–350g. were used. Litter-mates were allocated at random to one of the four treatment groups in each of the experiments. There were 10 rats in each treatment group. The larvae were from our own stock of *N. muris* which has been maintained for many generations in hooded rats. The methods for collecting, counting and placing the larvae on the skin of the rat for immunizing or test infection are as described by Leigh (1956) and Hunter & Leigh (1961). For larvae administered by other routes a tuberculin syringe was used (instead of a mercury pipette) with a needle for the intraperitoneal route and a polythene tube for the oral route. Records were kept of the number of survivors in each group and of the presence or absence of the skin reaction.

RESULTS

Table 1 shows the number of survivors and deaths in Expt. 1. No rats except those in Group IV showed a skin reaction. It therefore appears possible to produce an immunity without the skin reaction taking place. Table 2 shows the number of

Table 1. *Number of survivors and deaths in Experiment I*

	Group I	II	III	IV
Survived	2	9	10	10
Died	8	1	0	0

survivors and deaths in Expt. II. In the second experiment all the rats in Groups II–IV showed the skin reaction. The reaction in rats of Group IV was the most severe.

Table 2. *Number of survivors and deaths in Experiment II*

	Group I	II	III	IV
Survived	4	10	10	10
Died	6	0	0	0

DISCUSSION

Since the results show that a rat may be immune to *N. muris* without showing a skin reaction, this reaction is not a satisfactory test for immunity and the test will not be discussed further. However, there are two other points in the results worth noting. The first concerns the failure to get a skin response when the rats were immunized by the oral or intraperitoneal routes. This is interesting in the light of the work of Chase (1946) who found that the application of chemical allergens to the skin of guinea-pigs failed to elicit a skin reaction if the animals were previously given the allergens orally. The second point worth noting is the lack of positive evidence for the existence of a 'skin barrier'. In spite of the work of Chandler (1937*a*, *b*), there is still support for the idea that there is a skin barrier which prevents larvae penetrating the immune rat. If the skin played an important part in the resistance mechanism, then a lowered level of immunity could be expected when the test dose of larvae avoided the skin. Experiment I, Groups II and III, did not show any lowering of immunity. The only direct evidence that a skin barrier may exist, is the occasional finding of dead larvae in sections of the skin (e.g. Taliaferro & Sarles, 1939). The number of larvae found dead in the skin is such a small proportion of the total larvae given that this direct evidence is not very strong.

SUMMARY

Rats were given immunizing doses of *N. muris* larvae by three different routes: orally and intraperitoneally and through the skin. The immunized rats survived a test infection which was lethal for non-immune rats. Only the rats immunized by the skin route showed skin sensitivity.

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Studies on the resistance of rats to the nematode *Nippostrongylus muris* (Yokogawa, 1920)

III. Attempt to transfer immunity adoptively

BY G. C. HUNTER AND L. C. LEIGH
The Rowett Research Institute, Bucksburn, Aberdeen

(*Received* 7 *November* 1960)

It was first shown by Africa (1931) and Schwartz, Alicata & Lucker (1931) and confirmed by later workers (reviewed by Chandler, 1937; Taliaferro & Sarles, 1939) that rats could develop an actively acquired immunity to the nematode *Nippostrongylus muris* (Yokogawa, 1920). The rats were immunized with live larvae of *N. muris* and became resistant to subsequent infection. There is much recent evidence to show that lymphoid tissue from actively immunized animals can transfer immunity to normal animals of the same inbred strain. Examples of such transferred immunity are to be found in the works of Landsteiner & Chase (1942) and Chase (1945), for skin allergens; Mitchison (1954) for transplantable tumours; Harris, Harris & Farber (1954) for bacterial antigens and sheep red cells; and Billingham, Brent & Medawar (1954) for skin homografts. That state of immunity in which a normal animal becomes immune as a result of the transference of immunologically activated tissues has been called adoptively acquired immunity by Billingham, Brent & Medawar (1954).

The following experiments were designed to discover whether the actively acquired immunity in rats to *N. muris* could be transferred adoptively.

EXPERIMENT I

Lymph node transfer

Lymph node and spleen cells from actively immunized rats were to be transferred to non-immune rats. The recipient rats were then to be tested for immunity to *N. muris*, and evidence of immunity would be significantly fewer adult worms in the recipients than in the controls after a challenge dose of larvae.

Experimental design. There were three treatment groups:

Group I. The donors; rats with an actively acquired immunity against *N. muris*, to provide lymph node and spleen cells for Group II.

Group II. The recipients; rats which received lymphoid cells from Group I, and were later tested for immunity to *N. muris*.

Group III. Non-immune controls; which were tested for immunity to *N. muris* along with Group II.

The rats were allocated so that each recipient (Group II) was of the same sex, litter and substrain as its donors (Group I).

Materials and methods

The rats were 3 months old Wistar albinos from two substrains W3A and W3B, inbred for 70 and 66 generations respectively. The *N. muris* larvae used for immunizing the donors were from the Rowett stock maintained for many generations in hooded rats as described by Leigh (1956).

Immunization. The donors (Group I) were actively immunized by placing the following increasing doses of live larvae of *N. muris* on the skin of the abdomen at weekly intervals: 1050, 1845, 2883, 3811. The method of collecting and counting the larvae and infecting the rats has been described by Leigh (1956) and Hunter & Leigh (1961).

Transfer of lymph node and spleen cells. The methods of collection and transfer of lymphoid tissue have been described by Billingham, Brent & Medawar (1954) and Mitchison (1954). The donors were killed 14 or 15 days after the fourth immunization. The procedure thereafter was carried out under surgically sterile conditions. The rats were opened up, and the lymph nodes (as detailed below) and spleen were removed and put in a Petri dish containing Ringer's solution. The lymphoid tissue removed was—right and left axial nodes; right and left elbow nodes; superficial nodes of the neck (submaxillaries, major sublinguals, etc.); right and left anterior and posterior cervicals; right and left inguinals; intestinal node; cisternal group; right and left renal nodes; mesenteric nodes; thoracic nodes; lumbar nodes; caudal node; spleen.

The nodes and spleen were chopped with fine stainless steel scissors and pressed through a stainless steel sieve. The sievings were pipetted into 10 ml. conical centrifuge tubes and spun at 1400 r.p.m. on an M.S.E. minor centrifuge for 2 min. The supernatant was removed and the sediments collected and spun in a graduated centrifuge tube at 1400 r.p.m. for 2 min. and the supernatant discarded. The volume was read and 0·2 ml. was taken from the cell pack, after agitation, for estimating the number of cells transferred. The remainder was immediately injected into the recipient by the intraperitoneal route with a 5 ml. syringe.

Estimation of the number of cells transferred. The cell pack is fairly loose and may be broken up easily after spinning at the above speed. The 0·2 ml. sample removed with the graduated pipette was transferred to a 10 ml. graduated tube and made up to 10 ml. with Ringer's solution diluted 1 in 3 (1 vol. Ringer's/2 vol. dist. H_2O) to lyse any red cells present. After mixing, a sample was taken in a Neubauer counting slide and the number of nucleated cells in 80 (5×16) small squares counted. The volume and concentration of cells being known, the total number of nucleated cells given to each recipient was estimated and is shown with the number of donors in Table 1.

Test for adoptively acquired immunity. The recipients (Group II) and their controls (Group III) were tested for resistance to *N. muris*. Five or six days after the transfer of lymphoid cells each rat in Groups II and III was infected with $1277 \pm 199{\cdot}6$ larvae by the same method used for immunizing the donors. Nine days later all the rats were killed and the small intestines put in bottles containing 0·9% saline. The bottles were shaken and stored in the refrigerator at $-20°$ C.

until required for worm counts. It is characteristic of *N. muris* that the worms twist round each other, making accurate counting difficult. Shaking and maintenance at low temperature causes them to relax and facilitates accurate counting. The worms from the small intestine of each rat were counted under a steroscopic binocular microscope.

Table 1. *Worm counts and details of transfer*

	No. of worms recovered at autopsy		No. of nucleated cells given to recipients (millions)	No. of donors
	Controls	Recipients		
	1313	907	2482	5
	1192	1081	1780	4
	1237	1376	1683	3
	1132	1111	360	1
	1137	1170	1905	5
	1036	1225	1905	
	1336	1064	1587	4
	1236	1248	1423	3
	996	1072	1271	3
	1224	1338	2323	5
	1116	1269	879	3
	1188	1211	895	2
	1337	1246	841	2
	1205	1066	700	2
Total	16,685	16,384		
Mean	1192	1170		

Results

Table 1 shows the number of worms recovered from each control and recipient rat killed 9 days after receiving the test dose of larvae. The control count was 1·02 times as large as the recipient count and the 95% confidence limits were roughly 0·93 and 1·12; that is, the factor relating the control and recipient counts was not significantly different from 1·0. Adjacent to the recipients' worm counts in Table 1 is the estimated total number of nucleated cells received and the number of donors which provided these cells. There was no correlation between the recipients' worm count and the number of cells received. The results therefore show no evidence of transferred immunity.

In carrying out the foregoing experiment we had assumed that the rats were sufficiently inbred, that the transfer technique was satisfactory and that the donors were adequately immunized. The next four experiments were designed to show whether or not these assumptions were justifiable.

EXPERIMENT II

The use of skin graft to test whether the rats were sufficiently inbred

For successful adoptive immunization, the donor and host must be sufficiently inbred, so that donor tissues do not stimulate an immune response in the host.

The transfer may have failed because 66–70 generations of inbreeding were insufficient.

Skin, grafted between rats of the same highly inbred strain survives, whereas skin grafted between rats of little or no inbreeding breaks down. Billingham & Medawar (1951) and Billingham, Brent, Medawar & Sparrow (1954) have studied the breakdown reaction in mice and have shown that it is a sensitive test for non-compatibility. It has the advantage that the animals may be tested and the insufficiently inbred ones may be discarded before the experiment starts.

The 'ring' test of Billingham & Medawar (1951) was used to test the stock for compatibility. In this, the first rat gives a graft to the second, the second to the third, and so on, until the ring is completed by transplanting a graft from the last to the first. The skin grafting technique was that of Woodruff & Simson (1955). A 2×2 cm. square of skin, removed with a dermatome from the abdomen of one rat, was transplanted to a graft bed prepared on the side of the thorax of the next rat.

Results

In a ring of 58 animals from 10 litters and two substrains all the grafts were successful (Table 2).

Having got rid of unsuitable rats it might still not be correct to suppose that all rats with surviving grafts were sufficiently inbred for successful adoptive immunization. This and the transfer technique were tested simultaneously in the next experiment.

Table 2

No. of grafts	Particulars	Survival time to date of transfer (days)
45	Between litter-mates of same sex	70–143
10	Between rats of the same sex from different litters	78– 91
2	Between rats of same sex from different substrains (W3A and W3B)	79–108
1	Brother → sister transplant	108

EXPERIMENT III

To test the transfer technique

The transfer technique was tested by active and adoptive immunization with sheep red cells (S.R.C.). Adoptive immunity with this antigen has been shown by Harris, Harris & Farber (1954) in rabbits. Rats from the histocompatible stock were actively immunized with sheep red cells and their lymph node and spleen cells were transferred to other non-immune rats of the same strain. Evidence for successful transfer was the presence of sheep red-cell agglutinins in the recipients and a secondary response after a single injection of sheep red cells.

Preparation of sheep red cells. Sheep red cells from the same animal were used throughout. Blood was taken from the jugular vein of the sheep into an equal volume of Alsever's solution. The cells were spun and washed three times in 0·9%

saline, then the supernatant was removed and an equal volume of formol-saline was added (0·25 ml. formaldehyde (40 % soln.) in 99·75 ml. of 0·9 % saline).

Immunization. Active immunization of donor rats consisted of four intraperitoneal injections of 0·3 ml. sheep red cells; each rat was given one injection every fourteenth day. The lymph node and spleen cells of donors were transferred to the recipients 5 days after the last sheep red cell injection (the transfer technique as was used in the first experiment).

To evoke a secondary response, the recipients were each injected by the intraperitoneal route with 0·3 ml. sheep red cells 6 days after the transfer.

The recipients were bled before transfer, 3 days after transfer and at autopsy 13 days after transfer. These time intervals and amounts of antigen were determined by preliminary experiment to show a secondary response, if any, within the time limits set by the experimental life of *N. muris*.

The serum was collected, inactivated for half an hour at 56° C. and stored at −20° C. until wanted for the test.

Agglutination test. A simple quantitative agglutination test was used to demonstrate the level of sheep red-cell agglutinins. The antigen used was 0·2 ml. of the same formolized sheep red cells as those used for immunization of the donors, diluted in 9·8 ml. saline, i.e. 1/50; 0·5 ml. in each tube. The recipients' sera to be tested were diluted in 0·9 % saline, starting at 1 in 10 and doubling the dilutions up to 1 in 2560. In each row of tubes there was a saline control and a normal serum control which was the recipient's pre-transfer serum. Tubes were incubated in a water bath at 37° C. for 1 hr. and read 1 hr. later, after standing at room temperature.

Table 3. *Sheep red-cell agglutinin titres from an adoptively immunized rat*

be ...	1	2	3	4	5	6	7	8	9	Serum control	Saline control
rum lution ...	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	−	−
st- ansfer rum 3 days	++++	++	±	−	−	−	−	−	−	−	−
st- ansfer rum. After s.c. injection	++++	++++	+++	++	+	−	−	−	−	−	−

Results

Table 3 shows the agglutinin titres after such a test. Sheep red-cell agglutinins were found in the recipients' sera 3 days after transfer; and after injection with sheep red cells there was a secondary response which confirmed that adoptive immunity had taken place.

This experiment shows that the transfer technique was satisfactory, and it also confirmed Expt. II, showing that the rats are sufficiently inbred for adoptive transfer of immunity.

EXPERIMENT IV

Immunity of donors

Adoptive immunization also requires an actively acquired immunity in the donors. The immunizing doses of *N. muris* larvae used in Expt. I had always produced an actively acquired immunity in outbred hooded rats. This was our first experiment with inbred Wistar albinos and we noticed that these differed from hooded rats in the following ways:

(1) There was no skin reaction in the albinos.

(2) Some of the albino donors died under the anaesthetic at the third immunization and post-mortem examination showed that more worms were present than would be expected in immunized hooded rats.

(3) The albino donors barely maintained their weight during the 4 weeks. A gain would be expected in hooded rats.

(4) The recovery rate of adult worms from albinos was over 90% of the larvae given, whereas approximately 60% would be expected from hooded rats.

These differences made us doubt whether the donors had been adequately immunized.

A small experiment was designed: (1) to test the immunity of the immunized inbred albino; (2) to see if a skin reaction could be provoked in the albino; (3) to compare the albino with the hooded rat. Using the method outlined in Expt. I, the rats were given seven weekly infections of *N. muris* larvae, beginning at 500 and increasing to 14,000 per rat per week. A week later their immunity was tested by giving them a dose of larvae (22,000), which was known to be lethal for all normal rats.

Results

(1) The immunized albinos and hooded rats survived the test dose of larvae, whereas the non-immune ones died.

(2) The albinos showed a skin reaction after the 7th or 8th immunization; the hooded ones after the 5th or 6th immunization.

EXPERIMENT V

To determine whether the conditions discussed and tested above were satisfied when the rats were actively and adoptively immunised with Nippostrongylus muris

The plan was as in Expt. I, but the *donors* were tested for immunity to *N. muris* and the experiment was done *pari passu* with active and adoptive immunization with sheep red cells.

Design of experiment V. The treatment groups were:

Lymph node donors:

Group D1. Actively immunized against *N. muris* and sheep red cells.

Group D2. Actively immunized against sheep red cells only.

Controls for test of immunity to *N. muris*:

Group DC. Non-immune rats to compare with Group D1 to check that the test dose was lethal for all the normal rats.

Lymph node recipients:

Group R1. Recipients of lymph node and spleen cells from Group D1 donors.

Group R2. Recipients of lymph node and spleen cells from Group D2 donors.

Group RC. Controls for lymph node recipients Group R1. These were non-immune rats which received a challenge dose of *N. muris* larvae at the same time as Group R1 to show whether immunity had been transferred to Group R1 from Group D1.

Materials and methods

The rats, tested for compatibility by skin grafting in Expt. II were from the same stock as Expt. I, but belonged to the next generation (F67 and F71). They were allocated to the different groups so that the recipients were of the same sex and litter as the donors. In order to get enough donors (3 or 4 per recipient) some of the control groups DC and RC were taken from different litters. To avoid the possibility of a second set reaction, it was also arranged that the lymph node recipients were not graft recipients in Expt. II.

Immunization. Group D1 was actively immunized against *N. muris* by the method used in Expt. I, except that the course lasted 7 weeks (as in Expt. IV) instead of 4 weeks. Each rat received the number of larvae shown in Table 4. Sheep red cells were prepared and injected intraperitoneally as in Expt. III; each rat in Groups D1 and D2 received 0·3 ml. at the times shown in Table 4.

Table 4. *Immunizing schedule*

	Group D1		Group D2
Day	No. of larvae	Vol. of S.R.C. (ml.)	Vol. of S.R.C. (ml.)
1	487	—	—
7	1,167	—	—
14	1,491	—	—
21	2,485	—	—
24	—	0·3	0·3
28	3,525	—	—
35	7,000	—	—
38	—	0·3	0·3
42	13,050	—	—
49	22,900*	—	—
52	—	0·3	0·3
66	—	0·3	0·3

* Test dose.

Test for immunity of donors to Nippostrongylus muris

Obviously the worm count method of testing immunity of the recipients in Expts. I and V could not be used for the donors. The lethal dose method of Hunter & Leigh (1961) was therefore used instead as it is outlined below.

One week after the last immunizing dose each rat in Group D1 was given a dose of larvae (22,900) that was lethal for all normal rats. Survivors only were

considered immune and used as donors. To check that the test dose was lethal for normal rats, the same dose of larvae was given to each of the ten non-immune controls in Group DC.

In Group DC, the ten non-immune controls all died on the third or fourth day after receiving the test dose of 22,900 larvae. In group D1 there were 37 survivors out of 47 and these were used as donors for nine recipients. The deaths in Group D1 took place 7–14 days after the test dose.

Transfer. Lymphoid tissue was transferred from Groups D1 to R1 and D2 to R2, as in Expt. I, on the 15th day after Group D1 received the challenge dose of 22,900 larvae.

Adoptive transfer of immunity

(*a*) To *sheep red cells* was tested by demonstration of agglutinins as in Expt. III, where the preparation of sera and the agglutination technique were described.

(*b*) To *N. muris* was tested by the same method as Expt. I. Each rat in Group R1 and its non-immune control Group RC was infected with an average of 1075 larvae on the fifth day after the transfer of lymphoid tissue. Ten days later all rats in the two groups were killed for worm counts.

Results

Table 5 shows the number of worms recovered from each recipient (Group R1) and control (Group RC) rat killed 10 days after receiving the test dose of larvae. Again there was no evidence of transferred immunity. The control count was 1·07

Table 5. *Worm counts and details of transfer*

	No. of worms recovered at autopsy		No. of nucleated cells given to recipient (million)	No. of donors
	Controls	Recipients		
	824	876	4374·75	4
	973	819	4421·25	4
	1028	1010	4125·0	4
	1019	966	3137·5	4
	1090	1009	2065·0	4
	960	803	3902·5	4
	743	1016	4512·5	5
	1164	760	1880·0	3
	—	—	3351·25	5*
Total	7801	7259		
Mean	975	908		

* Recipient died after transfer.

times as large as the recipient count and the 95% confidence limits were roughly 0·90 and 1·28; thus, the factor relating the control and recipient counts was not significantly different from 1·00. There was no correlation between the worm counts of the recipients and the number of nucleated cells received. Weight loss from challenge to killing was examined, because non-immune rats usually show a

greater weight loss than immune rats. The control animals lost 4·4 g more than the recipients and the standard error of this quantity was ± 3·6 g. Therefore the difference between recipient and control was not significant. There was no evidence of a skin reaction to *N. muris* in the R1 recipients as a result of the transfer and the test dose of larvae. Table 6 shows the results of the agglutination tests on Groups R1 and R2. The end-points are shown as reciprocals of the serum dilution. At 3 days after transfer there were no detectable sheep red-cell agglutinins in the recipients whose donors had *N. muris* and sheep red cells; and this is consistent with the worm counts in showing no evidence of transferred immunity. Yet, agglutinins were present in the recipients whose donors had sheep red cells only. In spite of the absence of agglutinins at 3 days in the first group, subsequent injection of sheep red cells elicited a slightly greater response than the secondary response of the second group.

Table 6. *Titre of sheep red-cell agglutinins in the recipient rats*

	Bled 3 days after transfer	Injected with S.R.C. 6 days after transfer. Bled 15 days after transfer
Group R1. Donors given *N. muris* and S.R.C.	0	320
	0	640
	0	80
	0	640
	0	320
	0	320
	0	320
	0	640
Group R2. Donors given S.R.C. only	10	40
	20	640
	40	40
	40	160

DISCUSSION

Two attempts to produce adoptively acquired immunity to *N. muris* in rats have been made and have been unsuccessful. Experiments have shown that the rats were sufficiently inbred, that the technique was satisfactory and that the donors were immune to *N. muris*. One reason for the failure may be that the lymphoid tissue was immunologically inactive. This is suggested by the absence of sheep red-cell agglutinins 3 days after transfer in the recipients whose donors had *N. muris* and sheep red cells. Why this should happen when the donors get *N. muris* is not clear and it may be argued that the high agglutinin titre after injection with sheep red cells would not have occurred if the lymphoid tissue had been immunologically inactive. More experiments will be necessary to test this and also several other possible interpretations of the results. However, one fact emerges clearly: *N. muris* interferes in some way with antibody formation against sheep red cells.

SUMMARY

Two attempts to transfer immunity against *N. muris* to rats using the lymph node and spleen cells from actively immunized donors were unsuccessful. Experiments showed that the rats were sufficiently inbred, that the transfer technique was satisfactory, and that the donors were adequately immunized. The rats' competence as donors for adoptive transfer was demonstrated with sheep red cells as antigen. The adoptive transfer of immunity against sheep red cells was successful in recipients whose donors had sheep red cells only and no *N. muris*. There was no detectable transfer of immunity against *N. muris* or sheep red cells to recipients whose donors had received both.

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The collection of bird fleas from Fair Isle in 1956

By G. STANSFIELD
Museum of Natural History, Wood End, Scarborough

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In 1951 the laboratory examination of birds at Fair Isle Bird Observatory was extended to include the collection of ectoparasites. The fleas collected during the years 1951–55 have been recorded by Rothschild (1958).

In 1956 more intensive examination was carried out, and by 'de-fleaing' a higher proportion of the trapped birds and making an earlier start to the season, it was possible to collect more fleas in one year than in all the preceding years. The analysis of this material gives a more complete picture of the seasonal distribution of the three main species of flea amongst their bird hosts. This paper is concerned with these seasonal fluctuations and includes notes wherever the additional material throws further light on the previous work (Rothschild, 1958).

THE 1956 SEASON

Work commenced at the end of March. A total of 782 fleas was collected from some 3000 examinations, the method employed being identical with that used during previous years. No new fleas were identified, and the proportion of *Ceratophyllus garei* and *C. vagabundus* remained very much the same as it was in earlier years. Two specimens of *C. garei* were taken from a wheatear on 21 April, and the third from another wheatear on 5 May. The single specimen of *C. vagabundus* was taken from a rock pipit (a bird of the year) on 30 August. This bird had been negative for fleas when examined 6 weeks earlier.

Table 1. *Comparison of totals of fleas collected in the two periods*

	D. g. gallinulae	*C. gallinae*	*C. borealis*	*C. garei*	*C. v. vagabundus*	*C. fringillae*
1951–55	430	205	126	4	1	1
1956	328	198	252	3	1	—

Table 1 compares the flea totals for the two periods. Some of the differences may be discussed in terms of the general features of the season. The low proportion of *Dasypsyllus gallinulae gallinulae* in the 1956 totals is due in part to the poor spring migration (Table 2). Birds which in previous years had been the main source of *D. g. gallinulae* were less numerous, and those which were examined proved to have a lower infestation. A second factor with regard to *D. g. gallinulae* is the smaller number of wheatears examined in July when they are an important source of this flea. Prior to 1956, 380 wheatears were examined during July, whereas in 1956 the total was only 212.

The higher proportion of *C. borealis* may be in part explained by the April trappings. Eighty specimens were taken from wheatears alone before the end of this month.

Table 2. *Spring passage migrants as a source of* D. g. gallinulae

	1951–55			1956		
	Infested birds	Total examined	*D. g. gallinulae*	Infested birds	Total examined	*D. g. gallinulae*
Garden warbler	9	17	21	—	—	—
Willow warbler	24	33	24	2	6	3
Robin	30	58	31	2	16	—
Common redstart	22	29	28	1	1	1
Whitethroat	24	67	34	2	11	4

SEASONAL DISTRIBUTION

In the figures used below, the mean number of fleas per examined bird (in weekly periods) has been plotted in the form of a histogram. Miss Rothschild has used the proportion of birds carrying fleas as a measure of infestation, but, as there are more fleas per bird when more birds are infested, it is felt that a variable taking into account both factors helps to accentuate the maxima.

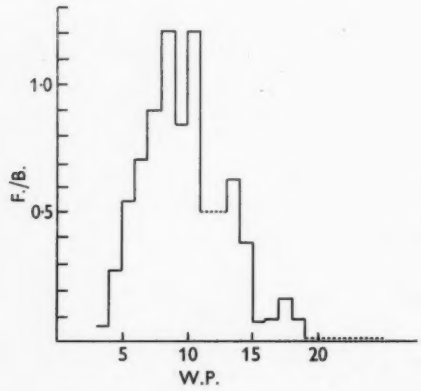

Fig. 1

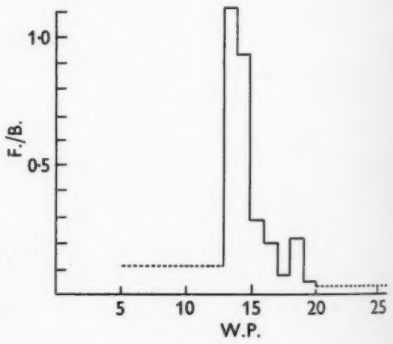

Fig. 2

Fig. 1. Infestation of wheatears by *Ceratophyllus borealis*. F./B., fleas per bird; W.P., weekly period, from 24 March.

Fig. 2. Infestation of rock pipits by *Ceratophyllus borealis*. F./B., fleas per bird; W.P., weekly period, from 24 March.

(1) *The wheatear* (Fig. 1)

Fig. 1 includes all original trappings of wheatears throughout the season. In the case of *C. borealis*, infestation rises steadily from the beginning of the season reaching a peak towards the end of May. It then appears to fall at the same rate, but the sequence is marred by a period between 9 June and 16 June when only

four birds were trapped. There is a small rise in infestation in July, when the first young birds are being trapped. *D. g. gallinulae* shows a similar trend with peaks at very much the same times as those shown by *C. borealis*, but the infestation is lower. *C. gallinae* is less common on this host and the figures available do not show any well marked maximum, although there is a higher infestation at the beginning of the season in May.

(2) *Rock pipit* (Fig. 2)

The adult birds of this species are not easily trapped in May and June, but those which were caught showed a low level of infestation. However with the first appearance of the young birds on 27 June, the infestation immediately reaches a peak and then falls rather quickly, rising to reach a smaller maximum at the beginning of August.

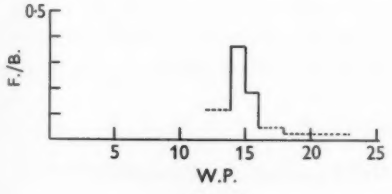

Fig. 3

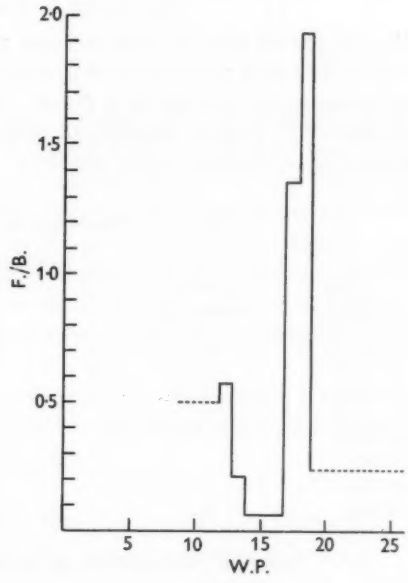

Fig. 4

Fig. 3. Infestation of meadow pipits by *Ceratophyllus borealis*. F./B., fleas per bird; W.P., weekly period, from 24 March.

Fig. 4. Infestation of starlings by *Ceratophyllus gallinae*. F./B., fleas per bird; W.P., weekly period, from 24 March.

The figures for the infestation of this species with *C. borealis* are inconclusive, except that infestation is highest in the first half of July. Only five specimens of *C. gallinae* were taken from this host.

(3) *Meadow pipit* (Fig. 3)

In 1956 the highest infestation of the meadow pipit with *D. g. gallinulae* occurs in early July, whereas Miss Rothschild found the peak in June. The first juvenile

meadow pipit in 1956 was trapped on 21 June, but the highest infestation occurs slightly later, when the bulk of the young birds were being caught.

Only four *C. gallinae* and three *C. borealis* were collected from this host.

(4) *Starling* (Fig. 4)

As in previous years, infestation was higher in the summer months, with two maxima coinciding with the appearance of the two broods of young. In Fig. 4, in which multiple infestations exaggerate the maxima, a much heavier infestation is shown for the second brood. There were certainly many more fleas available at this period, a starling on 27 July yielding twenty-nine fleas at one examination. However, in previous years Miss Rothschild found 'considerable variation in the degree of infestation of the second brood young'.

THE EARLY SPRING MIGRATION

As this period had not been covered previously all records are given in Tables 3 and 4. The high proportion of *C. gallinae* at this period is a new feature of the population study—in May it is far outnumbered by *D. g. gallinulae*. In 1956, the potential of *C. gallinae* appears to remain fairly constant until it rises with the appearance of the first young starlings.

Table 3. *Blackbird infestation in early spring*

Week ending	Birds examined	*C. gallinae*		*D. g. gallinulae*		*C. borealis*	
		♂	♀	♂	♀	♂	♀
31 March	49	.	3	3	1	.	1
7 April	6	2	2	.	.	1	.
14 April	2	.	.	1	1	.	.
21 April	9	.	.	4	.	1	.
28 April	.	.	.	.	.	.	.
5 May	4	.	.	1	.	4	1
12 May	.	.	.	.	.	.	.
19 May	4	.	1	2	1	2	.

Table 4. *Infestation up to* 30 *April* (*excluding blackbirds*)

Species	No. examined	*C. gallinae*		*D.g. gallinulae*		*C. borealis*	
		♂	♀	♂	♀	♂	♀
Starling	4	.	3	1	.	.	.
Wheatear	49	1	5	1	.	2	3
Meadow pipit	5	.	1	.	.	.	.
Willow warbler	3	.	.	.	2	.	.
Sparrowhawk	2	1	1	.	.	.	.
Robin	14	.	1	.	.	1	1

MATERIAL FROM RETRAPPED BIRDS

In Table 5 the fleas collected from retrapped and re-examined birds are listed for the season. The records under each month are of fleas actually collected during that month, although these may have been picked up by their hosts at any time

after the first examination. Records with more than 30 days between the two examinations are omitted from this table.

The 1956 material from May and June, which shows the three species in almost equal numbers, does not substantiate the previous work. It agrees, however, with the proportions taken from the early spring migrants in 1956. For the rest of the season the retrap material agrees with the previous figures, with peaks for *C. borealis* and *D. g. gallinulae* in July, somewhat later than the original infestations.

Table 5. *Fleas collected by re-infestation throughout the season*

	May		June		July		August	
	♂	♀	♂	♀	♂	♀	♂	♀
D. g. gallinulae	3	1	3	.	10	14	3	8
C. gallinae	1	2	1	1	3	1	5	5
C. borealis	.	1	3	2	3	4	2	2

CONCLUSIONS

The most important fact which arises from the 1956 collection is that *C. borealis*, *C. gallinae* and *D. g. gallinulae* are all available from at least the beginning of April. It is fairly clear from a comparison of the figures for 1956 and the previous years that there are a number of important discrepancies which make some of the earlier deductions doubtful. The total number of records would appear to be insufficient to obtain a clear picture, and it is doubtful whether more precise information can be gained by current methods. Seasonal differences must be great in view of the fluctuations of numbers and species of birds which migrate through Fair Isle.

It is suggested that the whole picture can only be built up by a consideration of individual host-flea relationships, and that the emphasis of future work should be upon the breeding biology of the flea species.

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The effect of the mite *Myocoptes musculinus* (C. L. Koch 1840) on the skin of the white laboratory mouse and its control

By D. P. WATSON, B.Sc., M.R.C.V.S.
Biology Department, Royal Free Hospital School of Medicine, London

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INTRODUCTION

The object of this work was to find the effect, if any, of the mite *Myocoptes musculinus* (C. L. Koch, 1840) on the skin of the white laboratory mouse by histological examination of normal and infected skins. It was undertaken because there is a good deal of research proceeding on mitotic activity using the malpighian layer of the skin of the mouse (Bullough, 1951, etc.). Previous work by Cook (1953), Stoner & Hale (1953), Madden, Tozloski & Sweetman (1954), Flynn (1955) and Davis (1957) gives no indication of the effect of the mite or of acaricides on the skin of the host.

Spread of infection

Infection of the bedding of the mouse rarely occurs or is accidental, and spread of the mite is from body to body in the nest.

M. musculinus has the third pair of legs in all stages modified for attachment to the hairs of the host by means of a specialized locking device (Watson, 1960). In the tritonymph and female, this device is also present on the fourth pair of legs. The mite is capable of rapid movement through the host hairs when this specialized limb is extended, but should the hairs be disturbed, it immediately attaches itself firmly. This reflex is a protective mechanism which prevents the mite being brushed off as the mouse travels through grass or bedding.

The young are infected from their mother at $4\frac{1}{2}$ days, the mites clinging to the vibrissae on either side of the mouth.

When a mouse dies, its body temperature drops and the mites leave the deeper hair layer and migrate to the hair tips. Here they attach themselves by means of the modified hindlegs, whilst the front ones wave continuously in the air and make grasping movements. When another mouse sniffs inquiringly at the body, the mite transfers to its vibrissae and so gains a new host. The grasping motions are continuous and purely reflex; the mite will grasp a steel needle if one is placed within reach.

Sites of infection

The usual sites of infection are face, between the ears, inguinal region and base of tail. In heavy infections on untreated mice, these are increased to include the shoulders and may spread right down the back. In the adult mouse, the presence of large numbers of mites causes poor greyish coat, some skin reddening, hair

thinning or loss, and some irritation resulting in localized skin bleeding (see Fig. 1). This is followed by increase in size of the affected areas, weight loss, marked emaciation, debility and eventually death of the mouse.

Histological examination of skin

An histological examination of the skin was made to determine the effect of the mite on the rate of cell division of the malpighian layer, and to see if this differed between young (6–8 weeks) and adult (6 months and older) mice. The mice used were all male and, as far as possible, were litter-mates, as Bullough & Johnson (1951*a*) found that the oestrus cycle and phase of hair growth affected mitotic activity.

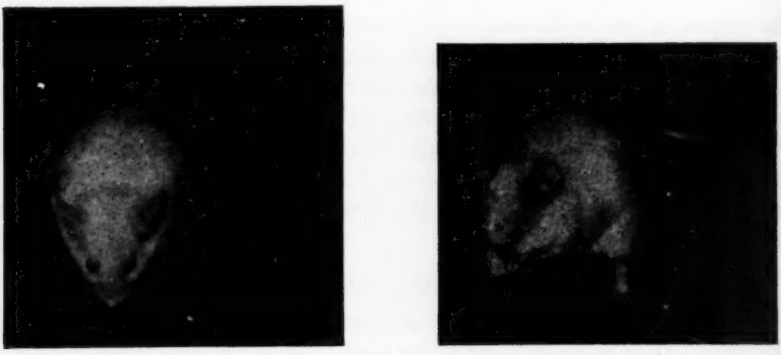

Fig. 1. Adult clean male mouse, coat white and glossy (left), adult male mouse showing signs of mange; note grey appearance of coat (right).

Samples of skin from snout, head and base of tail were treated with colchicine using the technique of Bullough & Johnson (1951*b*). The effect of the colchicine is to bring up the mitotic divisions of the cells in the malpighian layer to the metaphase stage and then arrest it. The samples were taken from three young clean and infected mice and three adult clean and infected animals. After embedding, the skin was sectioned at 5 and 8 μ and the sections stained in haematoxylin and eosin. The sections were examined histologically, and eighteen fields of view were selected at random from each sample and the number of metaphases counted in each. The results were subjected to statistical analysis. The skin was also examined to see if the mite had any other effect, for example, on the superficial layers of the epidermis and on the dermis.

Effect of Myocoptes musculinus *on the skin*

On examination of the sections, it was seen that, unlike *Sarcoptes scabiei*, which burrows into the skin, *M. musculinus* is purely a surface dweller. It feeds on the superficial epidermal layers and at no stage of its life cycle does it penetrate the deeper layers of the skin, or ingest blood or serum.

On comparing samples of skin from adult normal and infected mice, it was found that the infected skin had increased in depth (Fig. 2) and was twice as thick

as the normal in the region of the malpighian layer. There was no increase in thickness of the deeper layers, but there was, however, some cellular infiltration in the zone just below the malpighian layer. The cells which had invaded this region were mainly macrophages and some lymphocytes. Furthermore, the number of cells in the metaphase stage in the malpighian layer were found to be approximately four times as numerous in the infected as in the normal mouse (Table 1, para 1). In contrast to these results, the effect of *M. musculinus* on the actively dividing skin of clean and infected mice of 6–8 weeks, is negligible. The skin desquamation is quite marked at these ages and seems to more than supply the needs of the mite without having any pathological effect on the skin of the mouse. There is no increase in number of mitotic divisions and no noticeable cell infiltration (Table 1, para 2).

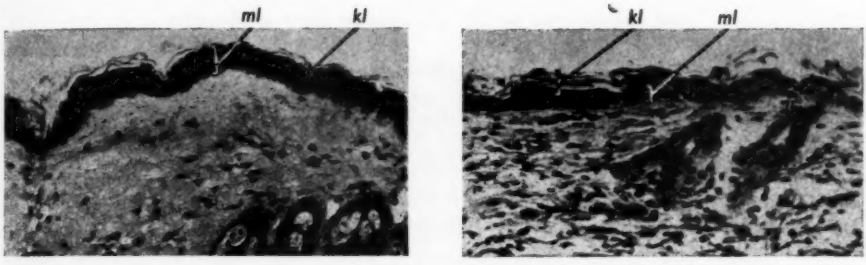

Fig. 2. Vertical section of mouse skin, infected (left), normal (right). Note the increase in depth of the malpighian layer and the nucleated cells formed by its division (*ml*) in the infected skin. The areas of the skin are from comparable regions of the body. Most of the superficial keratinized layers (*kl*) have been grazed off in the section of infected skin.

Table 1. *To show mitotic rate in clean and infected skin of mice of various ages*

Paragraph	Type	Mean mitotic count	S.E. of count	Difference of means	S.E. of difference means	Value of P for 't' test	Significance (positive if $P < 0{\cdot}01$)
1	Clean adult	2·16	0·0960	5·54	1·878	< 0·01	Significant
	Infected adult	7·7	1·876				
2	Clean young (6 weeks)	6·5	0·0707	0·25	0·2572	> 0·3	Not significant
	Infected young (6 weeks)	6·25	0·2474				

Control of Myocoptes musculinus

The following drugs were tested in an attempt to control the mite: benzene hexachloride (0·015 %), dieldrin (0·015 %), arsenic and sulphur (0·018 %), piperonyl butoxide (5 %) and dimethylphthalate (100 %).

Dimethylpthalate, an oily liquid, was found to be most efficacious, as it also controlled *Myobia*: a prostigmatid mite which also occurs on mice and is found

with the anterior half of its body buried in the superficial epidermal layers. Usually only one or two of these mites are present on a *Myocoptes*-infected mouse, but if only non-oily drugs are used to control the *Myocoptes*, the *Myobia* is unaffected by these and rapidly multiplies to become the chief parasite. Belozerov (1957) found that dimethylphthalate acted as a repellent against *Bdellonyssus bacoti*, rather than as an acaricide.

It seemed important to ascertain whether or not the drugs used had any effect, themselves, on the skin of uninfected mice. Histological examination of normal uninfected skin was carried out: (1) after one application of the drug; (2) after two applications, with a week's interval between them. It was then found that the acaricides did not affect the mitotic count in the malpighian layer (Table 2).

Table 2. *To show that the drugs used have no appreciable effect on the mitotic rate in the malpighian layer of the skin*

Type	Mean mitotic count	S.E. of count	Difference of means	S.E. of difference means	Value of P for 't' test	Significance (positive if $P < 0{\cdot}01$)
Clean undipped Adult	2·3	(1) 0·01	(1, 2) 0·22	0·08	$> 0{\cdot}0$	These differences are therefore not significant
B.H.C. dipped, × 2	2·08	(2) 0·0793				
Dieldrin dipped, × 2	2·46	(3) 0·0844	(1, 3) 0·16	0·09	$> 0{\cdot}01$	
Piperonyl butoxide dipped, × 2	2·07	(4) 0·0437	(1, 4) 0·23	0·26	$> 0{\cdot}01$	
Dimethylphthalate dipped, × 2	2·6	(5) 0·1054	(1, 5) 0·3	0·1054	$> 0{\cdot}01 < 0{\cdot}02$	

Effect of diet on symptoms of myocoptic mange

Laboratory mice are usually fed exclusively on rat cubes, which contain a concentrated diet. In infected mice fed on green food as well as cubes, symptoms of mange are delayed for periods of up to a year, although the mite population carried is as heavy or heavier than on those showing actual symptoms and fed cubes only.

This suggests that there is a precursor for protection in fresh green food, and several authors (György, 1938; Kartman, 1943; Kelemen & Tenyi, 1949) have suggested that vitamins, such as vitamin A and riboflavin, which are more abundant in fresh food, may help to protect animals from parasites and disease. That this protection is not merely a matter of acquired immunity is shown by the fact that adult mice infected with *Myocoptes* and deprived of green food, show symptoms of mange, and continue to do so at subsequent infections, the symptoms becoming more pronounced with each additional attack. Also, trace elements, such as iron, are found in soil-splashed Brassicas and are essential for the proper function of the thyroid, which also plays a part in combating disease (Reitler, 1947; Todd, 1949) and in preventing anaemia. Greens must be available for the mice at weaning time and supplied regularly each day, as the mice will nibble inquiringly at almost anything at this time. If greens are given to older mice previously fed only cubes, they refuse to eat them, their feeding pattern having been already fixed.

The rat cubes contain insufficient roughage for the proper functioning of the gut and post-mortem examination shows it to be always distended with gas. Thus the health of the laboratory mouse is very near the border-line and pathological symptoms may easily develop as a result of mite attack or even of treatment with acaricides.

Carriers of Myocoptes musculinus

Specimens of *Mus musculus* L. and *Microtus agrestis*, from various parts of the country, were examined for parasites. Only a few *Myocoptes* were found among the numerous parasites (other mites and lice) carried by the mice and no symptoms of mange were seen. It is thought that this may be due to the fact that these mice are hardier and, on an average, younger than the laboratory mouse. They also have access to a more balanced diet. No *Myocoptes* were found on *Apodemus sylvaticus*, and this confirms the observations of Elton, Ford, Baker & Gardner (1931).

SUMMARY

1. The means of attachment of the mite *Myocoptes musculinus* to the hair of the host, the mouse, and the sites where it is located are given.

2. Normal and skin infected with *M. musculinus* of young (6–8 weeks) and adult (6 months) mice is treated with colchicine and examined histologically.

3. Infected skin shows a mitotic count of approximately four times that of normal skin in the adult; there is no appreciable difference in the young.

4. Acaricides used to control the mite were benzene hexachloride (0·015%), dieldrin (0·015%), arsenic and sulphur mixture (0·018% arsenic), piperonyl butoxide (5%) and dimethylphthalate (100%); the latter being the most efficacious.

5. It is established that the drugs had no effect on the rate of division of the cells of the malpighian layer of the skin.

6. The number of *Myobia* present increases as the *Myocoptes* are killed unless dimethylphthalate, an oily liquid, is used at intervals.

7. Diet in conjunction with signs of mange is discussed.

8. Carriers of *M. musculinus* are given.

I wish to thank Cooper McDougall for supplying the materials used in dipping and swabbing, and the Infestation Control Laboratory of the Ministry of Agriculture for supplying me with a number of specimens of wild mice from different parts of the country. I am also grateful to the Council of the Royal Free Hospital School of Medicine for facilities for carrying out the work; and to Dr A. M. Hughes for her helpful guidance at all stages of the work and especially for reading the paper.

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Studies on the origin of the sticky coat on the eggs of the nematode *Thelastoma bulhõesi* (Magalhães, 1900)

By D. L. LEE*
The Molteno Institute, University of Cambridge

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Thelastoma bulhõesi (Magalhães, 1900), *Hammerschmidtiella diesingi* (Hammerschmidt, 1838) and *Leidynema appendiculata* (Leidy, 1850) are nematodes which live in the hind-gut of the cockroach *Periplaneta americana* L. All three species belong to the family Thelastomatidae, superfamily Oxyuroidea.

During an investigation into the nature and origin of the sticky coat on the eggs of *Thelastoma bulhõesi* it was noticed that two different secretions were present in the vagina: (*a*) a mucin-like substance in the vagina uterina which appears to form the sticky coat on the eggs, and (*b*) a refractive substance in the ovijector. This publication is concerned with the nature, origin and possible functions of these two secretions.

METHODS

The nematodes were killed and fixed in either Carnoy's fixative for 15 min. or in 10% formol saline for 24 hr. They were embedded in paraffin wax after dehydration in mixtures of ethyl alcohol and butyl alcohol. Transverse and longitudinal sections were cut at 5 μ. The histochemical techniques used in this investigation were taken from Casselman (1959), Gurr (1958) and Pearse (1953, 1960).

EXPERIMENTAL

(*a*) *The nature and origin of the sticky coat*

The eggs of *Thelastoma bulhõesi* were observed to stick to each other (Fig. 1) and to small particles of detritus when laid in 30% sea water, or in Hanks' saline, indicating that they possess a sticky coat. This coat, which swells when moistened, is normally very difficult to see; however, when females were induced to lay eggs in 30% sea water containing indian ink (1 drop of ink to 10 ml.) the small particles in the ink adhered to the sticky coat making it very obvious (Fig. 2). It was found that this sticky coat stained metachromatically (purplish red) with toluidine blue and immediately took on a blue-green colour in alcian blue (Fig. 1) showing that the coat contains acid mucopolysaccharide.

By opening the uteri of gravid females of *T. bulhõesi* in 30% sea water containing indian ink it was found that eggs in the lower part of the uterus possess a sticky coat (Fig. 2), whereas eggs in the upper region of the uterus are devoid of this coat. It appears that the sticky coat is deposited on the eggs in the uterus and becomes thicker as they pass farther down towards the vagina. The sticky coat

* Research Fellow of Christ's College, Cambridge.

gives a positive reaction with alcian blue which shows that it contains acid mucopolysaccharide and this indicates that the cells of the uterine wall secrete an acid mucopolysaccharide. Small amounts of material staining blue after Hale's test, and after alcian blue, have been observed in certain cells of the lower uterus wall and in the same sections the coat on the eggs stained positively. Large quantities of acid mucopolysaccharide are secreted by the vagina uterina (Fig. 3) in which the secretion is present in both the lumen and the cells lining the lumen (Figs. 4–6).

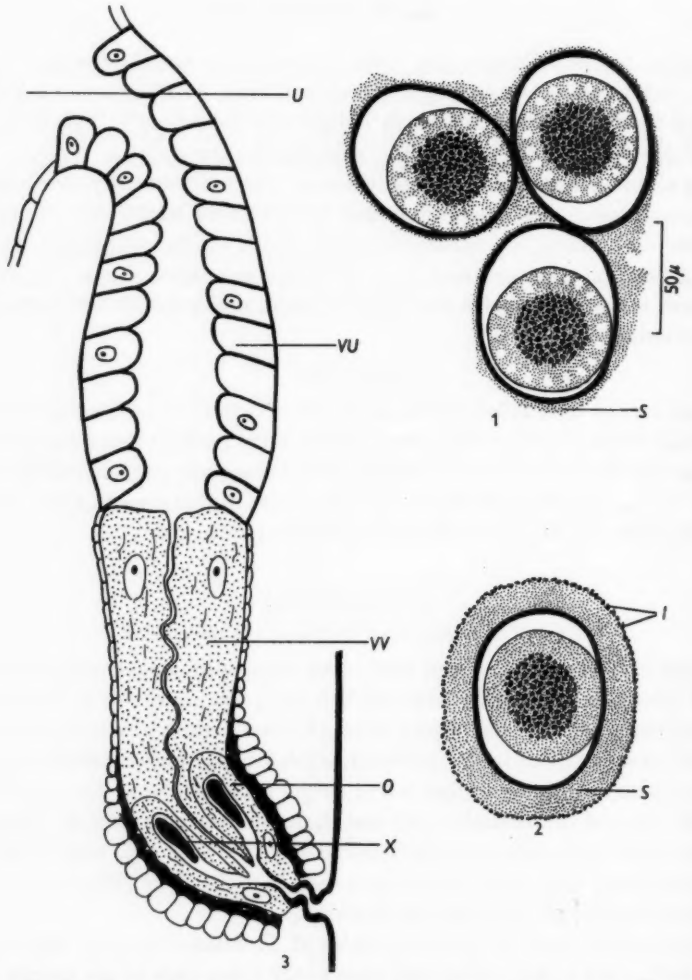

Fig. 1. Eggs of *T. bulhõesi* laid in 30 % sea water and stained with alcian blue to show the sticky coat.

Fig. 2. Egg from the lower part of the uterus with indian ink particles adhering to the sticky coat.

Fig. 3. Diagram to show the arrangement of the vagina and ovijector.

Neither the laid eggs, nor eggs removed from the uterus, of *Hammerschmidtiella diesingi* and *Leidynema appendiculata* possess a sticky coat, but the eggs of both of these species have a well developed outer coat of protein, which stains blue with mercuric bromo-phenol blue (Mazia, Brewer & Alfert, 1953). This protein coat is absent from the eggs of *T. bulhõesi*.

(*b*) *The nature and origin of the refractive substance in the ovijector*

Tests to detect protein in the sticky coat of the eggs of *T. bulhõesi* were negative; however, these studies have revealed an interesting refractive substance in invaginations of the lumen wall in the ovijector (Figs. 3, 6 and 7). These invaginations open into the lumen of the ovijector near to the vulva. The substance stains intensely with mercuric bromo-phenol blue and with aqueous bromo-phenol blue indicating that it is a protein. In both fixed and in living mature females of *T. bulhõesi* the refractive substance immediately stains orange with the diazo salt, Fast Red salt B (1 g. in 100 ml. of 20 % ethyl alcohol), which indicates that it is a phenolic substance. The substance in the ovijector also turns a dark brown after the Masson-Fontana ammoniacal silver technique which again indicates a phenolic substance; however, no positive results were obtained with Millon's test which is a histochemical test for phenolic substances. The Sakaguchi test for arginine, the ninhydrin-Schiff test and the dinitrofluorobenzene test for amino acids, Gibb's method for argentaffin granules and the performic acid-Schiff method for—SS—were negative. The substance stains intensely with iron haematoxylin and a characteristic greenish blue with toluidine blue. It gives no polysaccharide or lipid reactions.

The cells around the invaginations do not differ in their staining properties from the other cells of the vagina vera, and tests to determine areas of active protein synthesis by staining for RNA, using the pyronin-methyl green method, show no densely staining areas in the ovijector or vagina vera although the vagina uterina stains intensely. In some sections stained with toluidine blue a refractive substance, very faintly greenish blue, has been observed in the lumen of the vagina vera at its junction with the vagina uterina (Fig. 8) (the substance in the invaginations of the ovijector is refractive but stains a deep greenish blue with toluidine blue). It is possible, therefore, that this substance is secreted in the vagina uterina and passed along the lumen of the vagina into the invaginations of the ovijector where it changes in its staining properties. This theory is further strengthened by the fact that in some very young females, in which the gonads are still developing and in which no eggs have as yet formed, there is a refractive substance in the ovijector which does not stain with Fast Red salt B, although in females which are only slightly older (that is, the gonads are fully developed and one or two eggs are in the uterus) the substance does take up the stain.

When an egg is passing through the ovijector the latter becomes distended and the walls of the invagination become continuous with the wall of the lumen thus bringing the substance into contact with the egg (Fig. 9). This substance would appear to be passed out with the egg but I have been unable to detect it on laid eggs. If any of this substance is passed out the amount must be small as living

females of *T. bulhõesi*, stained with Fast Red salt B and kept in 30% sea water, still contained large amounts of the orange-stained substance after 2 days although the uterus had been almost emptied of eggs. I have been unable to detect an orange-stained coat on top of the acid mucopolysaccharide coat of eggs which had been in contact with this Fast Red salt B-stained substance whilst being laid.

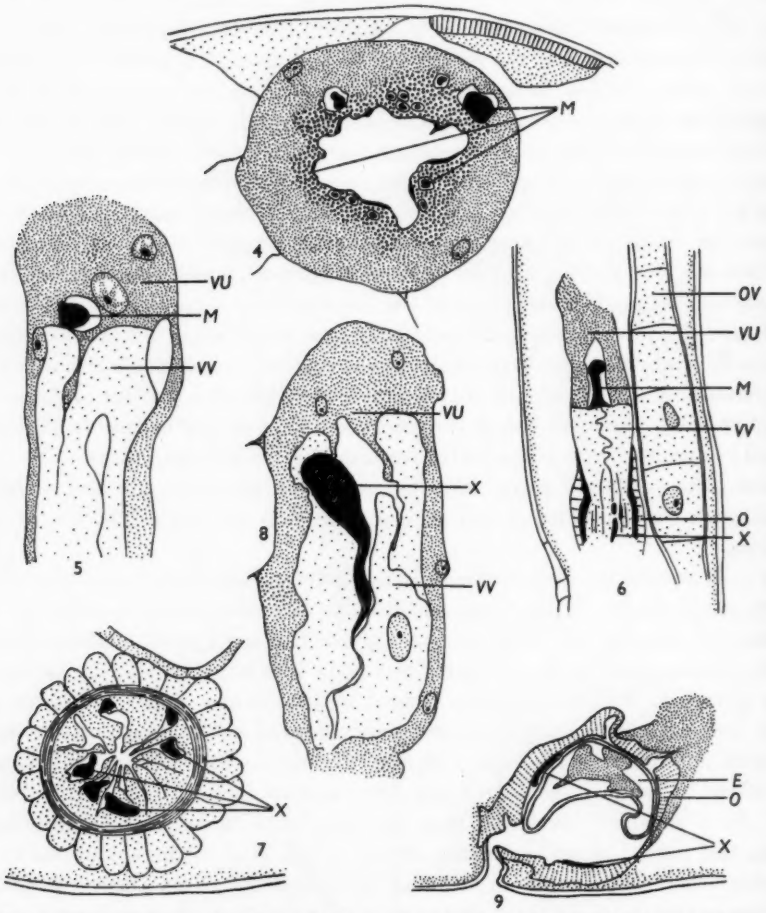

Fig. 4. Transverse section through the vagina uterina showing acid mucopolysaccharide in the walls.

Fig. 5. Longitudinal section through the vagina uterina and vagina vera.

Fig. 6. Horizontal longitudinal section through the region of the vagina, ovijector and vulva.

Fig. 7. Transverse section through the ovijector in the region of the invaginations.

Fig. 8. Longitudinal section through the vagina uterina and vagina vera to show the refractive substance in the lumen of the vagina vera at its junction with the vagina uterina.

Fig. 9. Longitudinal section through the ovijector containing an egg in the process of being laid.

Small amounts of a refractive substance have been observed in the ovijector of *H. diesingi* and this stains with mercuric bromo-phenol blue, but unlike the substance in the mature females of *T. bulhõesi* it does not stain with Fast Red salt B. There is no trace of this substance in the ovijector of *L. appendiculata*.

DISCUSSION

There are numerous references in the literature to nematodes which have sticky eggs and some of the terms used to describe the sticky coat have been 'mucoid', 'gelatinous' and 'albuminoid', but very few tests have been carried out to determine the nature of this sticky coat. In *Ascaris lumbricoides* it has been shown that the so-called 'albuminous' coat is not composed of albumin but is a jelly consisting of a protein and mucopolysaccharide provided with sulphuric acid residues (Monné & Hönig, 1954). The egg of *T. bulhõesi* also has a sticky outer coat which differs from that of *Ascaris*, in that it appears to be entirely composed of acid mucopolysaccharide, and from the eggs of *H. diesingi* and *L. appendiculata* which have an outer coat of protein and no sticky coat.

It has been shown in the present investigation that in *T. bulhõesi* acid mucopolysaccharide is secreted in the lower part of the uterus and in the vagina uterina. Presumably the fully formed eggs collect their sticky coat in the lower part of the uterus and in the vagina uterina.

The possession of a sticky coat on the egg would appear to have several advantages for the survival of the eggs and of the species; the eggs will tend to stick together and so the host will in all probability ingest more than one egg; the eggs will stick to the feet of the cockroach and will be ingested when the insect cleans itself with its mouth parts; the eggs would appear to resist drying for longer periods than eggs without this coat as the coat would have to dry out completely before moisture was lost from the embryo; and if moisture became available after a dry period the coat would rapidly imbibe it and swell up again.

It is well known that many nematodes belonging to the family Thelastomatidae lay their eggs in strings or in clumps of two or three (Basir, 1956), and in many cases it is obvious that the substance holding the eggs together is more than just acid mucopolysaccharide but seems to contain threads as well. It is possible that in these nematodes the threads are formed by a protein substance in the ovijector similar to that found in *T. bulhõesi* as described above but that in *T. bulhõesi* this substance is unable to form these threads.

SUMMARY

1. The eggs of *Thelastoma bulhõesi* are shown to have a sticky outer coat of acid mucopolysaccharide.

2. The acid mucopolysaccharide is secreted by the lower uterus wall and by the vagina uterina.

3. A protein substance of unknown function has been observed in invaginations in the ovijector of *T. bulhõesi*.

4. This protein substance appears to be secreted by the lower part of the vagina uterina and passed along into the ovijector.

5. Possible functions of the mucopolysaccharide coat on the eggs, and of the protein substance in the ovijector, are discussed.

I wish to express my thanks to Dr P. Tate for many invaluable suggestions and for his constant encouragement during this investigation.

KEY TO LETTERING ON FIGURES

E = egg
I = ink particles
M = acid mucopolysaccharide
O = ovijector
OV = ovary
S = sticky coat
U = uterus
VU = vagina uterina
VV = vagina vera
X = protein substance of unknown function

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Studies on the egg of the horse bot-fly, *Gasterophilus intestinalis* (De Geer)

By R. J. TATCHELL
Molteno Institute, University of Cambridge

(*Received* 3 *December* 1960)

Pantel (1913) distinguished two main types of eggs of Diptera. They were differentiated by the possession of 'thick' or 'thin' walls. The 'thin-walled' variety were further characterized by the columnar nature of the chorion. The columnar structure was studied in greater detail by Wigglesworth & Beament (1950, 1960), who concluded that the spongy protein of the columns and the inner layer contains air which supplies the respiratory needs of the egg. Although Hinton (1959, 1960) demonstrated that air could fill the spaces between the columns, Wigglesworth & Beament (1960) showed that this need not necessarily be the normal situation. Wigglesworth & Beament (1950) described the structure of the chorion in *Calliphora*, *Drosophila* and *Syrphus*, and Davies (1948) showed that the chorion of *Lucilia* was devoid of chitin and was probably mainly composed of a slightly tanned liproprotein. Beament (1946) showed that the chorion of the egg of *Rhodnius* was composed of seven layers of protein material differing in their degree of tanning, vulcanization and impregnation with lipid. Despite the presence of lipoproteins the chorion, in the main, was permeable to water; the egg being waterproofed by a layer of wax on the inner polyphenol layer of the resistant endochorion. The resistant protein of the endochorion was also shown to contain air which was in communication with the atmosphere via the pseudomicropyles and thus able to supply the respiratory needs of the egg. Wigglesworth & Beament (1950) describe similar respiratory adaptations in the eggs of other insects.

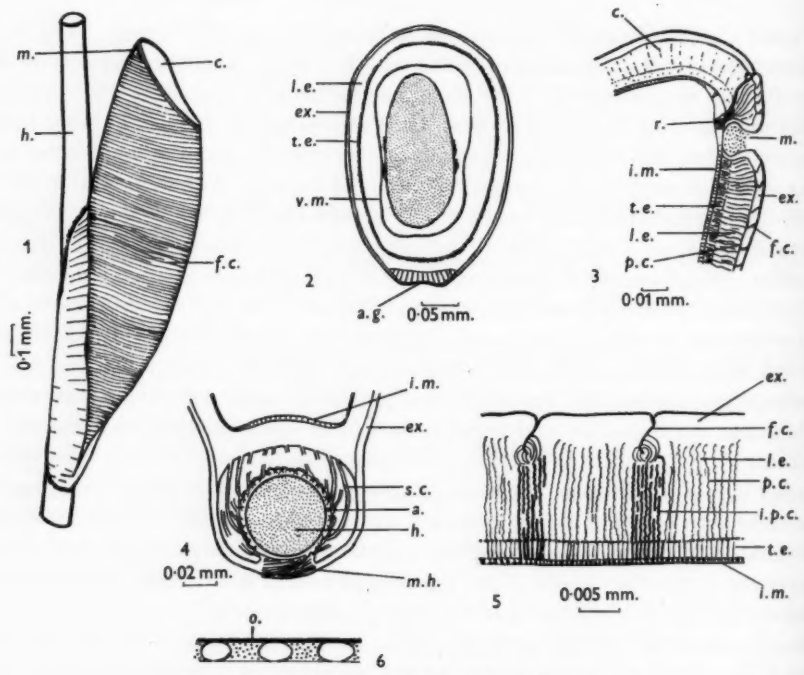
In the course of a study of *Gasterophilus intestinalis* it was noted that the eggs were thick walled and in consequence they were studied in greater detail for comparison with other dipterous eggs.

MATERIALS AND METHODS

Eggs, containing fully formed 1st-instar larvae, were obtained from the hides of freshly slaughtered horses. Developing eggs were also obtained from pupae and imagines, but not with sufficient reliability to enable a complete study of the formation of the egg membranes. The staining and histochemical reactions of the chorion were investigated with unfixed frozen sections, paraffin sections after fixation in Bouin's solution and by frozen sections of post-chromed formol-calcium fixed material for lipid constituents (Baker, 1946). Frozen sections were cut at 10–15 μ and paraffin sections at 5 μ. The respiratory adaptations were studied by the injection techniques of Wigglesworth (1950, 1959).

RESULTS

Description of egg of G. intestinalis. The eggs are white, approximately elliptical in outline and slightly flattened laterally (Fig. 1). They are attached singly to the hairs of the host by dorsal extensions of the chorion enclosing the hair for three-quarters of the length of the egg. The average dimensions of the egg are 1·3 mm. in length with a maximum diameter (dorsoventral) in cross-section of 330 μ. The larvae hatch by forcing off the distinct cap found at the free end of the egg facing outwards away from the hair. The cap is oval with a long diameter of 300 μ. There



Figs. 1–6. The egg of *Gasterophilus intestinalis*.

Fig. 1. The egg of *Gasterophilus intestinalis* attached to a hair.

Fig. 2. Transverse section through the anterior part of the attachment organ of the egg of *G. intestinalis*.

Fig. 3. Longitudinal section of the chorion through the micropyle and the junction with the cap.

Fig. 4. Transverse section through the posterior part of the attachment organ.

Fig. 5. Longitudinal section through the chorion after injection with cobalt sulphide using Wigglesworth's technique.

Fig. 6. Section of vitelline membrane (diagrammatic).

is a single micropyle (diameter 10 μ) situated dorsally at the free end of the egg. The surface of the egg is very finely grooved transversely giving it a shagreened appearance under low magnification.

Structure of the unspecialized chorion. The chorion is composed of a number of layers of mainly protein material. Although all the layers give a positive reaction to the xanthoproteic test for proteins, other tests and staining reactions reveal differences in the compositions of the layers. In section (Figs. 2, 3, 5) there is an outer translucent exochorion approximately 4 μ in thickness. The transverse grooves, which represent the boundaries between the transverse rows of follicular cells, are confined to this layer and slope inwards at an angle of 50°. The grooves terminate in a short reflexed section which, although bounded by exochorion, projects a short way into the middle layer of the chorion. Beneath the exochorion is the lipoprotein layer of the endochorion. This layer, which is 11 μ thick, contains numerous helical pore canals running at right angles to the surface. The pore canals are filled with air, which, by an interference effect, gives the eggs their white colour. The third layer of the chorion, the tanned endochorion, is 2 μ in thickness and contains the inward continuations of the pore canals which have decreased in diameter and show a straightening of the helix. The inner surface of the tanned endochorion is covered with a large number of minute projections which support the excessively thin (*c.* 0·2 μ) inner membrane of the chorion. The gap between the inner membrane and the tanned endochorion measures 0·5 μ except in the region beneath the attachment to the horse's hair where it is increased to 1·0 μ.

The staining and chemical reactions of the inner membrane were difficult to determine with certainty and the following account of these reactions is mainly concerned with the outer layers. With aqueous 1% acid and basic fuchsin the exochorion and tanned endochorion showed a strong basophilia whereas the lipoprotein endochorion was acidophilic. Similarly, only the lipoprotein layer was stained by light green and showed β metachromasia with toluidine blue. With the Gram–Weigert modification of Gram's technique the liproprotein layer stained very lightly and the tanned endochorion more intensely. Haematoxylin, after mordanting with ferric alum, produces an intense lake in the exochorion and tanned endochorion and also the inner membrane.

The distribution of phenolic compounds was investigated by staining with ammoniacal silver nitrate solution (Lison, 1936). The exochorion and tanned endochorion gave a strongly positive reaction, being stained a golden-brown, whereas the lipoprotein and inner membrane were stained faintly. In the case of the inner membrane, owing to its thinness, this probably indicated the presence of considerable amounts of polyphenol material. The distribution of polyphenols was not changed by methylation (0·2N-HCl in methanol) for 24 hr. at 60° C., although, as would be expected, the existing sites stained much more intensely with ammoniacal silver nitrate solution. Confirmation that this staining was caused by the presence of polyphenols was given by the absence of any reaction after 15 hr. benzoylation (10% benzoyl chloride in dry pyridine) at 60° C., which is known to destroy phenolic groups.

The susceptibility to tanning of the chorionic layers was studied by treating sections for 18 hr. in the dark with saturated aqueous solutions of benzoquinone. The exochorion and tanned endochorion were faintly stained while the lipoprotein

layer and the inner membrane appeared to be unaffected. This indicated that the layers were almost fully tanned or that the reactive groups were blocked.

Monné & Hönig (1954) have shown that quinone-tanned protein may also undergo keratinization, and Beament (1946) quotes a number of cases where considerable amounts of sulphur have been found in insect eggshells. Sections were therefore stained by the performic-acid-Schiff (PFAS) technique (Pearse, 1960) to demonstrate —SS— linkages. A distinctly positive reaction occurred in the exochorion and the tanned endochorion. As the PFAS technique is also specific for unsaturated lipids a series of sections of post-chromed formol-fixed material was treated with Sudan black B. None of the layers was stained, and in consequence free lipid must be absent from the chorion (or at least in layers visible with the light microscope). The presence of sulphydryl groups was investigated by the ferricyanide technique of Chevremont and Frederic (Pearse, 1960) and the nitroprusside technique of Hamnett and Chapman (Pearse, 1960). With the first a negative result was obtained but with the nitroprusside test a faintly positive reaction was noted in the exochorion.

Although the test for free lipid had proved negative the acid haematin test (Baker, 1946) for phospholipins was applied and intense staining resulted in the lipoprotein and tanned endochorion. However, the same reaction occurred in the pyridine-extracted controls. The acid haematin reaction is also given by nucleoproteins but a negative Feulgen test and the failure of the endochorion to stain selectively with methyl green or Pyronin Y indicated that RNA and DNA were absent. Therefore, the intense staining with acid haematin must be due to the retention of the stain by a lipoprotein complex which can resist pyridine extraction. This was confirmed by chemical tests and by the selective staining of the lipoprotein endochorion with luxol fast blue.

A negative result with the PAS technique in all the layers of the chorion indicated that polysaccharides were probably not present. In the case of chitin this was confirmed by the solubility of all the layers in heated caustic potash. All the chorionic layers reacted positively to the xanthoproteic test but the ninhydrin test was positive only in the lipoprotein layer and Millon's reaction was given only by the exochorion.

The constitution of the chorionic layers was further studied by means of their reactions with inorganic reagents. The chorion was extremely resistant to solution in cold, concentrated mineral acids (nitric, hydrochloric and sulphuric acids) and cold, saturated solutions of potassium hydroxide. However, with concentrated sulphuric acid small amounts of a clear insoluble material were extruded from the follicular grooves. Wigglesworth (1947) has shown this to be a convenient method for demonstrating the cement layer of the epicuticle of *Rhodnius*. Therefore, it is possible that the eggs of *Gasterophilus* contain a minute accumulation of cement in the follicular grooves. With saturated KOH at 60° C. there is a slow general breakdown but at 150° C. dissolution is complete within 20 min. When heated with concentrated nitric acid, or with cold concentrated nitric acid saturated with potassium chlorate, the exochorion, the tanned endochorion and the inner membrane dissolved completely, whereas the lipoprotein layer was broken down to

liberate large numbers of oil droplets. The same reaction was observed after extraction with chloroform and thus confirms that the layer is composed of a lipoprotein complex. As the outer layer was broken down the cement material of the follicular grooves remained undissolved although slightly swollen.

The work of Goddard & Michaelis (1934) has shown that keratin could be dissolved by sodium sulphide and sodium thioglycolate solutions by the destruction of the disulphide cystine linkages. In the case of the chorionic layers of *Gasterophilus* the exochorion and tanned endochorion dissolved readily in solutions of sodium sulphide. The lipoprotein layer was more resistant; much debris remaining after 24 hr. at 60° C. This confirmed the histochemical findings with respect to the distribution of —SS— linkages.

The exochorion and tanned endochorion were readily dissolved by a 10% solution of sodium hypochlorite, a characteristic of quinone-tanned proteins with little or no combined lipid.

Structure of the egg cap. The cap (Fig. 3) is composed of layers, similar in thickness and staining reactions to those of the unspecialized chorion. However, the follicular grooves of the exochorion and pore canals of the endochorion are not present. The endochorion is not completely homogeneous but contains faint vertical striations. In eggs where the larvae are fully developed and ready to hatch the cap, although closely applied to the rim of the egg opening, appears to be held only by the inner membrane of the endochorion (Fig. 3). At the junction between cap and shell this membrane is slightly thickened and is supported on a series of ridges running around the inside of the rim, instead of the columnar supports. These ridges are 1·0 μ in height and thus there is a line of weakness across the wide gap where the membrane support changes from the innumerable short columns of the cap to a few ridges. At the time of hatching vigorous thrusts from the larva would tear the membrane at this point and displace the cap.

The micropyle. There is a single micropyle, 10 μ in diameter, which in 'mature' eggs penetrates as far as the inner membrane of the endochorion. The walls of the micropylar pit are composed of exochorion 3 μ in thickness. The pit is plugged with a substance which stains intensely with alcian blue and therefore can be identified as an acid mucopolysaccharide. In the unfertilized egg this plug is more diffuse and extends some way out of the pit.

The region of attachment. The posterior two-thirds of the dorsal surface of the chorion is specialized to form a structure which firmly grips the shaft of the horse's hair. Anteriorly (Fig. 2) the structure consists of a groove lined with an adhesive substance on a sculpted protein layer supported on stout columns (2·0 μ in diameter) arising from the lipoprotein endochorion. Laterally the groove is bounded by extensions of the exochorion. Posteriorly (Fig. 4) these lateral extensions become progressively larger and finally overlap to completely surround the hair. Where the extensions overlap they are continued as a fringe of very fine hairs (*c.* 0·5 μ in diameter) which are further embedded in adhesive material. The lining of the groove continues to be supported on numerous columns from the lipoprotein layer of the endochorion and as the lateral extensions enlarge so the length of these columns increases from 5 μ to approximately 60 μ, with an appropriate

curvature. The columns enlarge distally and join up to form the lining of the attachment groove. This layer is clearly marked into hexagonal areas, representing the original follicle cells, each with a central depression associated with each column, and is coated with an adhesive substance. This adhesive has a negative reaction with the PAS technique, alcian blue and Sudan III and IV; it therefore has no affinities with some mucins or lipids. However, it stains positively with aqueous Bromo-phenol-blue which is a diagnostic test for basic proteins (Johri & Smyth, 1956). The columns contain longitudinally running spaces which give them a spongy appearance in section. It is not possible to inject cobalt sulphide or kerosene–olive oil mixture (Wigglesworth, 1950, 1959) into these spaces and they must be either fluid filled or, if air-filled, not in communication with the atmosphere.

The columns stain very faintly with ammoniacal silver nitrate solution and are probably only very slightly tanned. Attachment of the egg to the hair will be effected by the hair being clamped by the overlapping extensions of the tanned exochorion against the adhesive coated inner layer supported on soft protein columns.

So far as the formation of the attachment organ is concerned it would appear that a tube-like invagination is formed from the posterior part of the dorsal wall of the follicle. This opens out anteriorly and gradually disappears as the unspecialized chorion is laid down anteriorly. When the egg is fully formed the infolded follicular cells produce, by secretion or degeneration, the basic protein which is assumed to be adhesive in function.

Water relations. The free 1st-instar larva loses water rapidly and soon shrivels and dies when removed from the egg. However, while within the egg membranes it shows remarkable resistance to desiccation. Thus, at room temperature (*c.* 20° C.) a batch of eggs survived for 7 weeks in a desiccator, over phosphorus pentoxide. Visible signs of water loss were noticed only after the death of the larvae which, in any case, occurred at the same time as in those eggs maintained on damp blotting paper. Despite this, the layers of the chorion can be shown to be readily permeable to water. After immersion in aqueous basic fuchsin for 10 min. the exochorion was strongly stained and there were signs of infiltration at the rim. After 2 hr. the air had been displaced from most of the pore canals and the tanned endochorion was strongly stained. Similarly, the lipoprotein layer stained intensely when the egg was immersed in aqueous acid fuchsin. Treatment with chloroform, before and after abrasion with aluminium oxide, for 1 min. at room temperature, was without effect on the ability to withstand desiccation in those eggs where the larvae were not killed immediately. There was no indication of quicker penetration by alcoholic basic fuchsin. It seemed likely therefore that any waterproofing layer must be associated with the inner membrane of the endochorion or the vitelline membrane. In this connexion it was noted that the surface of the inner membrane, unlike the surface of the exochorion, retained its hydrofuge properties even after prolonged immersion in water. This need not necessarily indicate the presence of a wax layer as highly tanned proteins also possess marked hydrofuge properties. However, when eggs from which the larvae had hatched were immersed in aqueous stains to just below the rim there was never

any accumulation of stain within the egg cavity. Therefore there must be a waterproofing layer associated with the inner membrane. Attempts were made to remove the cap, thus rupturing the inner membrane, and exposing the vitelline membrane. In most cases the larvae were able to free themselves by piercing the membrane. The operation was carried out successfully with one egg which was then placed over P_2O_5 in a desiccator at approximately 20° C. The larva remained alive, as was seen by small muscular twitches, for 37 days without there being any obvious signs of marked water loss. After the death of the larva shrinkage was rapid and after 3 days it was completely shrivelled.

Christophers (1960) described a technique for estimating the critical temperature at which the wax of eggs of *Aëdes* lost its waterproofing properties. The eggs were immersed in saturated solutions of sodium chloride at graded temperatures and the amount of 'dimpling' noted after 2 hr. Dimpling did not occur in the eggs of *Gasterophilus*; the chorion became translucent when saturated, and the pore canals filled with the saline and it was possible to assess the amount of shrinking of the larva within the vitelline membrane. By this means it appeared that the critical temperature was between 50 and 60° C.

Respiratory adaptations. Wigglesworth & Beament (1950, 1960) have shown that in many insect eggs the respiratory requirements are supplied by a layer of air, free or held by a thin layer of spongy protein, lining the shell and connected to the atmosphere by a variety of means. In *Gasterophilus* Wigglesworth's injection technique showed that there was a layer of free air in the space between the inner membrane and the tanned endochorion. From Fig. 5 it can be seen that this layer of air communicates with the atmosphere by means of specialized pore canals running to the follicular grooves. Where the pore canals run through the material of the exochorion their bore is very greatly reduced and in untreated sections they resemble a diffraction effect around the groove. These pore canals as they run through the lipoprotein and tanned endochorion are in no way distinguishable from the other pore canals. Injected material also reaches the free-air layer by means of the gap between the rim and the cap of the shell. From its appearance after the olive oil–kerosene injection technique it is possible that the inner membrane may also contain air.

The vitelline membrane. In the mature egg this structure measures approximately 0·8 μ in thickness. When stained it contains numerous clear circular areas which in section have the appearance of flattened droplets retained by an excessively thin inner wall (Fig. 6). The remainder of the membrane is composed of an homogeneous tanned protein. The clear areas failed to react with any of the staining techniques available. In the unfertilized egg, immediately after removal from the adult fly, the membrane is much thicker, measuring 3–4 μ. The clear areas appear to be represented by faintly differentiated droplets which, in section, are unflattened. If the eggs are removed from the female and allowed to dry out at room temperature the vitelline membrane assumes the appearance it has in the normal fertilized egg.

DISCUSSION

As with *Rhodnius*, *Lucilia* and *Aëdes* the chorion is completely non-chitinous, being composed of layers of tanned protein and lipoprotein. Two of the chorionic layers also show a degree of vulcanization, or keratinization, in the presence of —SS— groups linking adjacent protein chains. The staining and chemical reactions suggest that the protein of the outer and inner layers has undergone complete tanning so far as the theoretical primary condensation of free amino-groups with quinones is concerned. The absence of free amino-groups in the exochorion and tanned endochorion is confirmed by the negative result with the ninhydrin test. The pale yellow colour of the chorion, when the pore canals are free of air, suggests that little secondary polymerization of protein chains by cross-linkage formation has occurred. This tends to be confirmed by the very faint staining with the PFAS technique for —SS— groups. The lipoprotein endochorion which is only lightly tanned is, however, heavily impregnated with lipid.

Other dipterous eggs have been shown to be intolerant of low humidities. Thus *Lucilia* (Davies, 1948) at 30° C. gave only a 68 % hatch with 25 % relative humidity. In the present investigation eggs of *Calliphora* were placed over P_2O_5 in a desiccator at 20° C. After 2·5 hr. 60 % of the eggs showed, by slight dimpling, that considerable water loss was taking place. This was so in all the eggs after a further 1·5 hr. and 40 % were grossly distorted. Both *Lucilia* and *Calliphora* have eggs of Pantel's 'thin-walled' variety; in the case of *Lucilia* the thickness of the chorion is 5·0 μ and in *Calliphora* it is 2–3 μ. The egg of *Gasterophilus* is of the 'thick-walled' variety and there is a marked resistance to desiccation. However, this is not because of the thickness of the chorion as the visible layers were shown to be permeable to water. This is, of course, similar to the situation in *Rhodnius* were the chorion is permeable with the exception of the wax layer covering the inner surface. Although it was shown that the vitelline membrane possessed a highly efficient waterproofing layer there was also shown to be a primary waterproofing layer on the inner membrane of the endochorion. From the appearance of the egg while immersed in saturated saline it is possible that the critical temperature of the primary wax layer was close to 50° C. whereas that of the vitelline membrane was closer to 60° C. According to Wigglesworth (1945) it would seem that a critical temperature of 50° C. is normal for insects requiring the ability to withstand dry environments. The critical temperature for the secondary wax layer on the vitelline membrane is quite high although not as high as that of the secondary layer of the egg of *Rhodnius* which is 65° C. That of *Calliphora* was shown, in the present investigation, to be in the region of 40° C. which is very similar to that of the larval waterproofing layers. As it has been noted that the higher the critical temperature of an insect's wax layer the greater is its ability to withstand desiccation, it is likely that this factor is decisive when comparing eggs of the 'thick-' and 'thin-walled' varieties. As has been shown by Wigglesworth (1945) and Beament (1959) a layer of air between two cuticular layers forms a highly efficient mechanism for reducing transpiration. It is possible, therefore, that this is a valuable additional function for the layer of free air between the tanned layer and inner membrane of the endochorion.

It was interesting that the columnar organization of the chorion, so obvious in the eggs of higher Diptera, was also present in *Gasterophilus*. Although very small in proportion to the rest of the chorion it provides a free-air space of approximately 0·5 μ in thickness surrounding the egg and in direct communication with the atmosphere. The attachment to the horse's hair, with its outer lamina supported on columns, is also a variant of this pattern which has structural advantages as well as being economical of material.

SUMMARY

1. A description is given of the egg of *Gasterophilus intestinalis*.

2. The chorion has been shown to consist of a number of tanned protein and lipoprotein layers.

3. The waterproofing of the egg is shown to be dependent on a primary wax layer on the inner membrane of the endochorion and a secondary layer on the vitelline membrane.

4. The respiratory requirements of the egg are supplied from a free-air space between the inner membrane and the tanned protein layer of the endochorion which communicates with the atmosphere by specialized pore canals opening into the follicular grooves.

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KEY TO LETTERING

a.	adhesive layer
a.g.	attachment groove
c.	cap
ex.	exochorion
f.c.	follicular groove
h.	hair
i.m.	inner membrane supported by minute columns
i.p.c.	injected pore canals
l.e.	lipoprotein endochorion
m.	micropyle
m.h.	marginal hairs of attachment organ
o.	outer surface of vitelline membrane
p.c.	pore canals
r.	ridges supporting inner membrane at junction of rim and cap
s.c.	soft protein supporting columns
t.e.	tanned endochorion
v.m.	vitelline membrane

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A new nematode *Pharyngodon morgani* sp.nov., intestinal parasite of a lizard, *Mabuya striata*, in Nyasaland

By W. M. FITZSIMMONS, F.R.C.V.S.
Commonwealth Bureau of Helminthology, 103 *St Peter's Street St Albans, Herts*

(*Received* 31 *December* 1960)

Family OXYURIDAE Cobbold, 1864
Subfamily Oxyurinae Hall, 1916
Genus **Pharyngodon** Diesing, 1861

Definition. Oxyurinae—mouth with three small lips; cuticle with or without lateral flanges; vestibule absent; oesophagus with a posterior globular bulb containing a valvular apparatus and separated from the rest of the oesophagus by a slight constriction; excretory pore behind the oesophageal bulb; posterior extremity of the body in both sexes obliquely truncate ventrally at the level of the anus, rounded and prolonged as a long subulate tail which is provided with spines or smooth.

Male. The posterior extremity varies from a condition in which the caudal alae may be present and supported by one pair of pre-anal papillae which are usually sessile and two pairs of pedunculated post-anal papillae; through a condition in which only one pair of post-anal papillae supports the caudal alae, to a condition in which caudal alae are absent and all the papillae are sessile or entirely absent (*P. apapillosus* Koo, 1938); the spicule is imperfectly chitinized or absent.

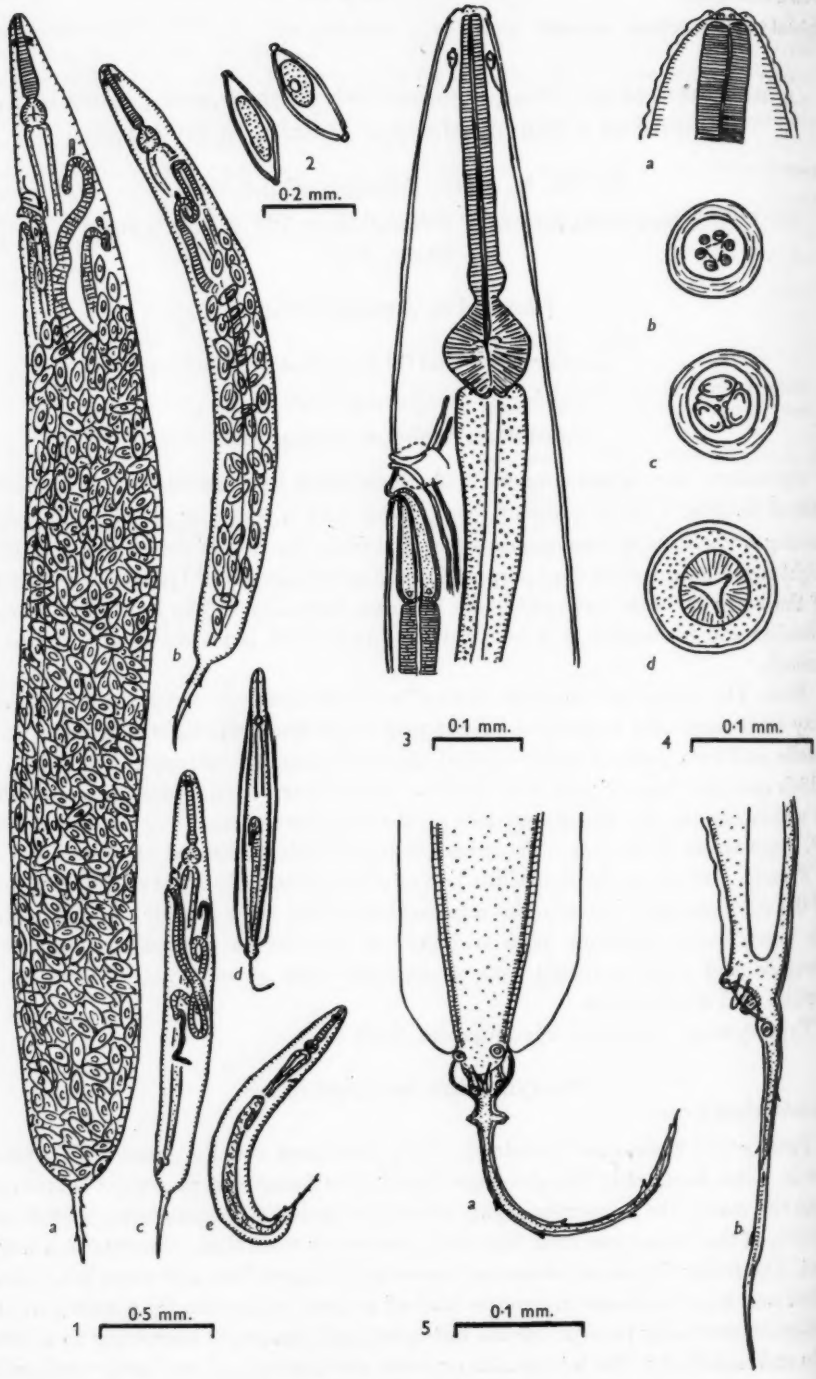
Female. Anus near the end of the body a short distance in front of the beginning of the subulate tail; vulva in the anterior half of the body usually slightly behind the conspicuous excretory pore and pre- or post-bulbar. Oviparous, eggs very elongate and oval, provided with a cuticular knob at each pole. Parasites of reptiles and amphibians.

Type species: *P. spinicauda* Dujardin, 1845.

Pharyngodon morgani sp.nov.

General description

Twenty-five hosts were examined. Both sexes were usually present in the host, but in some hosts only females were found. The females were always commoner than the males, the proportion being about five to one. The nematodes were found in 70 % of the hosts examined, but there were never more than 12 worms in a single host. The living female is robust and somewhat maggot-like, and when it has been killed and fixed its shape resembles that of a cigar, being bluntly rounded at the posterior extremity (except for the tail-spike) and gradually narrowing to a truncate end anteriorly. The lateral alae or crests are narrow and are barely noticeable



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in the female. The male is much smaller than the female and bears conspicuous lateral alae and small inconspicuous caudal alae.

The head is not demarcated from the rest of the body and is flattened anteriorly. Three lips surround the mouth opening, each of which bears two papillae. On the dorsal halves of the two subventral lips there is, in close proximity to one of the papillae, an amphid. Just behind the anterior margin of each lip, on its inner side, there is a small tooth-like protrusion. There is no vestibule or pharynx. The oesophagus consists of a fairly short, straight corpus, which swells very slightly before it is joined to the spherical bulb by a constricted isthmus. The intestine is slightly swollen anteriorly and straight, and its junction with the rectum is marked by the presence of three rectal glands. The excretory pore is post-bulbar in both sexes; in the male it lies behind the oesophageal bulb at a distance rather less than the total length of the oesophagus and its bulb; in the female it lies a little farther forward; the pore is a transverse slit surrounded by a chitinous rim which is produced internally and partially supports the walls of the excretory vesicle; in the female the pore lies immediately in front of the vulva. The nerve ring surrounds the oesophagus in its anterior third in both sexes.

Male. The posterior end of the male is usually ventrally curved. There are well developed lateral alae which originate in the anterior half of the worm at about the level of the mid-point of the oesophageal corpus and extend back, steadily widening, so that at their greatest width the sum of the widths of the two alae exceeds the width of the body at the same level. The alae narrow slightly near their termination and are attached to the body in such a way that there is a short free lobe at their most posterior limit, which is at the level of the most anterior or precloacal pair of papillae. The caudal alae, which are rather narrow, begin immediately behind the lateral alae and run back to end just behind the post-anal pair of papillae, so that the most posterior, or caudal, pair of papillae is not included in them. The papillae are all sessile and there are, as has been indicated above, three pairs of them, one pair being pre-anal, one pair post-anal and one pair caudal. The tail-spike usually bears from three to six spines. No spicule is visible, but there is a ventral prolongation of the cloaca flanked on either side by a rod-like structure and extending backwards to the level of the pre-anal papillae. The testis is single and extends anteriorly for nearly half the length of the body and is then reflected for a short distance.

Fig. 1. (*a*) Mature female, old, greatly distended with eggs, lateral view. (*b*) Mature female, young, uteri filled with eggs, but body not yet distended, ventral view. (*c*) Immature female, arrangement of genitalia visible, no eggs, lateral view. (*d*) Mature male, ventral view showing lateral alae. (*e*) Mature male, lateral view.

Fig. 2. Eggs.

Fig. 3. Anterior end, female, lateral view.

Fig. 4. (*a*) Head end, female, dorsal view. (*b*)–(*d*) End face views of head: (*b*) superficial, (*c*) and (*d*) at progressively deeper planes of focus.

Fig. 5. (*a*) Posterior end, male, ventral view, showing arrangement of lateral and caudal alae, papillae, ventral prolongation of cloaca with rod-like structures, and spines on tail. (*b*) Posterior end, male, lateral view.

Female. The female is widest at about the middle of the body, that is to say, at some distance behind the level of the vulva. Behind the anus the tail narrows abruptly and is continued as a tail-spike which bears from nine to eleven spines. The relative length of this spike diminishes with the increasing maturity of the female. The vulva lies in the anterior half of the body closely behind the excretory pore. Its anterior lip protrudes somewhat and its opening has the form of a transverse slit. The vagina (sometimes referred to in the literature as the ovejector) is a muscular, highly contractile organ which runs backwards from the vulva parallel to the body wall. Its junction with the common body of the uterus is clearly demarcated. The uterus continues in the same direction as the vagina. Its common body is muscular and appears to assist the expulsion of the eggs. In immature females one of the two much convoluted branches of the uterus runs forwards and the other backwards, each to continue as an elongated ovary. The eggs are characteristic of the genus and have a cuticular knob at either pole.

Specific diagnosis

Male. Length 1·53–1·97 mm., average 1·69 mm.; maximum width 0·11–0·17 mm., average 0·14 mm.; length of oesophagus plus bulb 0·26–0·29 mm., average 0·27 mm.; diameter of oesophageal bulb 0·06–0·07 mm.; length of rectum 0·08 mm.; head–nerve ring distance 0·08–0·09 mm.; head–excretory pore distance 0·46–0·54 mm., average 0·50 mm.; cloaca–tail tip distance 0·27–0·34 mm., average 0·29 mm.; total number of papillae on caudal extremity, 3 pairs; caudal alae do not include posterior pair of caudal papillae; greatest width of lateral alae 0·031 mm.

Female. Length 3·24–5·78 mm., average 4·42 mm.; maximum width 0·34–0·61 mm., average 0·46 mm.; length of oesophagus plus bulb 0·46–0·50 mm., average 0·48 mm.; diameter of oesophageal bulb 0·11 mm.; length of rectum 0·11–0·17 mm., average 0·15 mm.; head–nerve ring distance 0·11–0·14 mm., average 0·12 mm.; head-excretory pore distance 0·57–0·83 mm., average 0·69 mm.; head–vulva distance 0·65–0·90 mm., average 0·76 mm.; anus–tail tip distance 0·65–0·80 mm., average 0·73 mm.; dimensions of egg 0·140 × 0·033–0·146 × 0·037 mm.; tail, 9–11 spines.

Host. Mabuya striata (Peters).

Location. Large intestine.

Locality. Dedza and Blantyre, Nyasaland.

Types. In the London School of Hygiene and Tropical Medicine.

Affinities. This species is easily recognized as belonging to that group of the genus *Pharyngodon* in which (1) the caudal alae of the male do not include the posterior pair of post-anal papillae; (2) the anterior pair of post-anal papillae is single, not forked; and (3) the tail of the female bears spines. In this group there are three other species: *P. extenuatus* (Rudolphi, 1819) from North African and Spanish lizards, *P. oxkutzcabiensis* Chitwood, 1938, and *P. giganticus* Read & Amrein, 1953, both from North American lizards. A spicule is present in *P. extenuatus*, but is absent from the two American species and from the new species here described. *P. giganticus* is very similar in many respects to the new species but can be differentiated by its size and its relatively shorter oesophagus and by

the fact that its egg is relatively smaller and bears a cuticular knob at one pole only. The new species also resembles *P. oxkutzcabiensis* very closely, but can be separated from it by the distance of the excretory pore from the oesophageal bulb, by the width of the lateral alae, by the presence of the tail spines in the male and by the number of spines on the tail of the female.

This new species is named *Pharyngodon morgani* in honour of the late Dr D. O. Morgan of the Cambridge School of Veterinary Medicine.

The generic diagnosis by Yorke & Maplestone (1926) as revised by Malan (1939) is slightly modified in this paper to accommodate *Pharyngodon apapillosus* Koo, 1938 and *P. medinae* Calvente, 1948.

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Dichelyne trionyxi sp.nov. and *Dichelyne wallagoni* sp.nov., two new species of the family Cucullanidae Cobbold, 1864

By G. K. CHAKRAVARTY and G. MAJUMDAR
Department of Zoology, University of Calcutta, India

(*Received* 4 *January* 1961)

When specimens of the turtle, *Trionyx gangeticus*, and of the fish, *Wallago attu*, were purchased in a local market and examined, nematodes were found in the intestines of both these hosts. Two males were found in the intestine of the turtle, and a male and three females in the intestine of the fish. Examination of these nematodes showed that they all belonged to the genus *Dichelyne* Jägerskiöld, 1902, but that none of them belonged to any known species of this genus. The genus *Dichelyne* was regarded by Yorke & Maplestone (1926) as being synonymous with the genus *Cucullanus*, O. F. Mueller, 1777, but was later re-instated as a distinct genus by J. F. Mueller (1934), who reduced the genus *Dacnitoides* Ward & Magath, 1916, to synonymy with *Dichelyne*. In the light of these emendations the genus *Dichelyne* now includes the following species: *D. fossor* Jägerskiöld, 1902, 1909; *D. cotylophora* (Ward & Magath, 1916), J. F. Mueller, 1934; *D. robusta* Van Cleave & Mueller, 1932; *D. fastigatus* Chandler, 1937; and *D. diplocaecum* Chandler, 1937.

To these must now be added the two new species described in this paper. The name *D. trionyxi* has been given to the species found in the turtle, *Trionyx gangeticus*. The name *D. wallagoni* has been given to the species found in the fish *Wallago attu*. These are the first records of species of the genus *Dichelyne* from any host in India and *D. trionyxi* is the first record of any species of this genus in a reptilian host in any country, all the other species having been found in fish.

Dichelyne trionyxi sp.nov. (Figs. 1, 2)

Host. *Trionyx gangeticus*.
Location. Intestine.
Locality. Calcutta.

Description

The worms are slender, elongated and have a knob-like head. The cuticle of the anterior end of the body is conspicuously annulated and finely striated. The anterior end is straight and not bent dorsally. The cuticle is continuous around the anterior margin of the head and is longitudinally striated. The mouth is a dorso-ventral slit bounded by two lateral lips, each of which has three papillae. There is no buccal capsule. A pseudo-capsule formed by the dilatation of the anterior part of the oesophagus is present. The oesophagus is also enlarged posteriorly so that it has a club-shaped appearance. A short, narrow, ventral caecum arises from the intestine just behind the oesophagus and passes forwards.

There is no oesophageal appendix. The posterior end of the male tapers gradually behind the cloaca. There is a pre-anal muscular sucker without a chitinous rim. The posterior end is slightly alate. There are eleven pairs of caudal papillae, six of which are post-anal and five pre-anal. The arrangement of the pre-anal papillae varied in two specimens observed. In one specimen all the pre-anal papillae were behind the muscular sucker, while in the other one pair of these papillae was in front of the sucker. The spicules are fairly long, subequal and gradually taper to the tips. There is a trough-shaped accessory piece just in front of the cloacal aperture.

Measurements in mm.

Body: length, 8·85–13·71; breadth, 0·31–0·46.
Head: length, 0·39–0·52; dorso-ventral diameter, 0·39–0·49.
Oesophagus: length (including pseudo-capsule), 1·0–1·3; breadth (posterior part), 0·14–0·18.
Pseudo-capsule: length, 0·39–0·45; breadth, 0·33–0·39.
Nerve ring: distance from the anterior end, 0·41–0·53.
Excretory pore: distance from the anterior end, 0·83–1·0.
Intestinal caecum: length, 0·19–0·28; breadth, 0·039–0·052.
Tail: length, 0·27–0·32.
Pre-anal sucker: distance from the cloaca, 0·35–0·55.
Spicule: length—left, 1·9–2·3; right, 1·8–2·2.
Accessory piece: length, 0·026; breadth, 0·039.

Affinities

D. trionyxi differs from the other species as follows: *D. cotylophora* is smaller, as a dorsal intestinal caecum, but no accessory piece and the number and arrangement of the caudal papillae of the male are different. According to Chandler (1937) the male of *D. robusta* has no muscular sucker. This character alone is sufficient to differentiate it from *D. trionyxi*. *D. fastigatus* resembles *D. trionyxi* in that it has the same number of papillae on the lips and on the caudal region of the male and in having an accessory piece; the accessory piece of *D. fastigatus* is, however, longer than that of *D. trionyxi* and its body and spicules are shorter. Further, the arrangement of the caudal papillae in the male is different and it has no pre-anal muscular sucker. *D. diplocaecum*, described by Chandler (1937), differs from *D. trionyxi* in having two intestinal caeca; but this species was founded on examination of female specimens only and the features of the male are at present unknown.

In view of all these differences the name *D. trionyxi* is proposed for the species found in the turtle, *Trionyx gangeticus*.

Diagnosis of *D. trionyxi* sp.nov.: the presence of grossly annulated and finely striated cuticle; two lateral lips each with three papillae; a pre-anal muscular sucker; six pairs of post-anal and five pairs of pre-anal papillae; slight caudal alae in the male; subequal spicules; trough-shaped accessory piece. Female unknown.

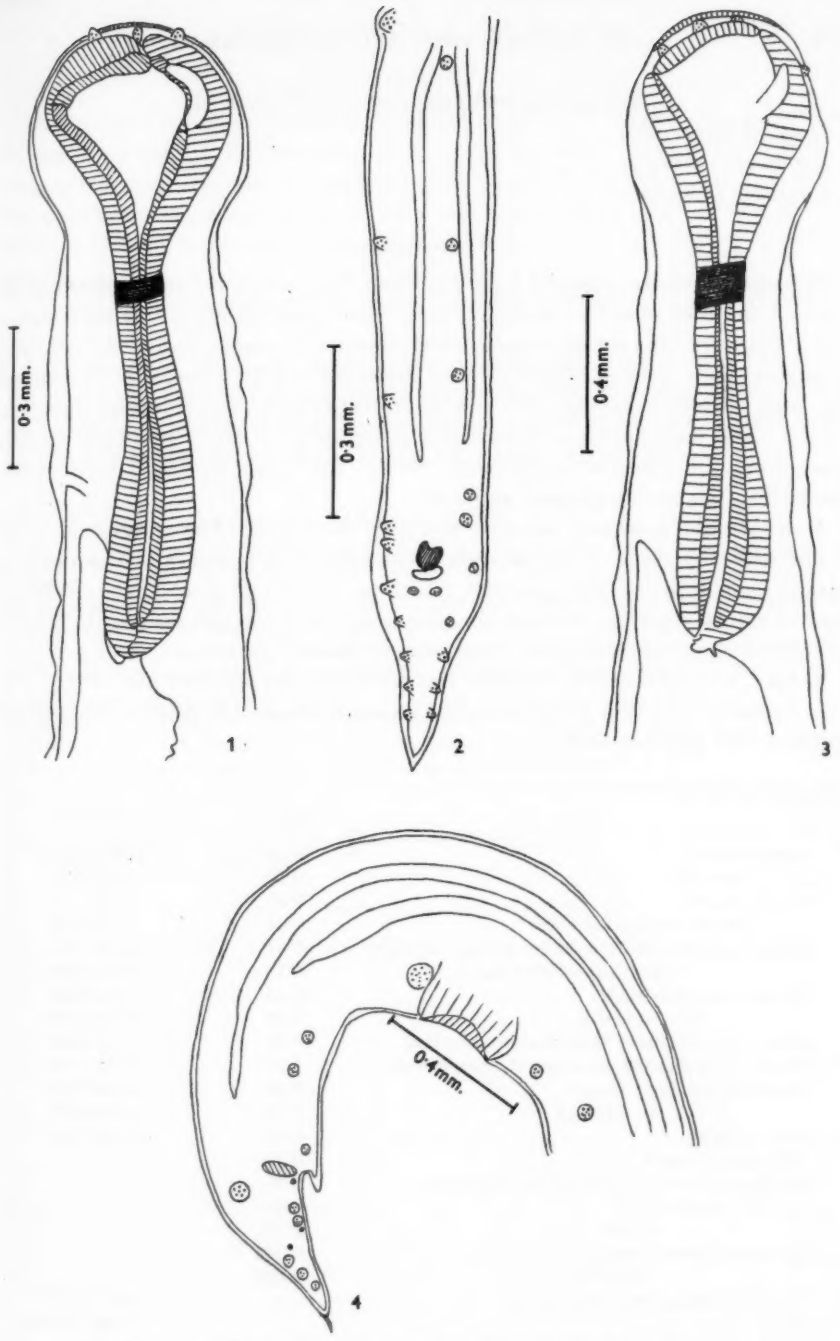

Fig. 1. *D. trionyxi* sp.nov. Male: anterior end, lateral view.
Fig. 2. *D. trionyxi* sp.nov. Male: posterior end, ventral view.
Fig. 3. *D. wallagoni* sp.nov. Female: anterior end, lateral view.
Fig. 4. *D. wallagoni* sp.nov. Male: posterior end, lateral view.

Dichelyne wallagoni sp.nov. (Figs. 3, 4)

Host. *Wallago attu.*

Location. Intestine.

Locality. Calcutta.

Description

This species has an enlarged knob-like head. The cuticle is finely striated on all parts of the body, but the annulations on it are more prominent at the anterior end of the body. The cuticle is continuous around the anterior end of the head and is longitudinally striated. The mouth is a dorso-ventral slit bounded by two lateral lips, each with four papillae. There is no buccal capsule, but a pseudo-capsule is present. The oesophagus is club-shaped posteriorly. A short intestinal caecum arises from the intestine immediately behind the oesophagus and extends forwards. There is no oesophageal appendix.

Male. The tail is conical and ends in a very short spike. The anterior lip of the cloaca is very prominent and extends backwards. A pre-anal muscular sucker is present, but it has no chitinous rim. There are no caudal alae. There are fifteen pairs of caudal papillae, of which six are pre-anal and the rest post-anal. The two spicules are long and subequal. An accessory piece is present.

Female. The tail is acutely conical. The vulva is in the posterior half of the body. Two vulval lips are very prominent. The vagina is directed forwards. The uteri are opposed. The eggs are oval.

Measurements in mm.

	Male	Female
Body: length	15·48	13·00–15·83
breadth	0·50	0·39–0·52
Head: length	0·41	0·45–0·52
dorso-ventral diameter	0·46	0·46–0·61
Oesophagus: length (including pseudo-capsule)	1·37	1·19–1·24
breadth (posterior part)	0·18	0·16–0·20
Pseudo-capsule: length	0·45	0·39–0·46
breadth	0·39	0·36–0·39
Nerve ring: distance from the anterior end	0·57	0·52–0·58
Excretory pore: distance from the anterior end	1·00	0·94–1·00
Intestinal caecum: length	0·32	0·26–0·28
breadth	0·07	0·06–0·07
Tail: length	0·35	0·28–0·32
Tail spike: length	0·02	—
Pre-anal sucker: distance from the cloaca	0·40	—
Spicule: length—left	2·40	—
right	2·17	—
Accessory piece: length	0·065	—
breadth	0·026	—
Vulva: distance from the tail tip	—	4·80–6·20
Egg	—	0·052 × 0·039

Affinities

The features which differentiate *D. wallagoni* from *D. cotylophora*, *D. robusta*, *D. fastigatus* and *D. diplocaecum* are the same as those which differentiate *D. trionyxi* from these species. *D. wallagoni* differs from *D. trionyxi* in that it is larger, the male has no caudal alae, the number and arrangement of the caudal papillae of the male are different and the cloaca of the male of *D. trionyxi* has no prominent anterior lip.

In view of these differences the name *Dichelyne wallagoni* is proposed for this species.

Diagnosis of *D. wallagoni* sp.nov.: grossly annulated cuticle with fine striae; two lateral lips each with four papillae; no caudal alae; male with fifteen pairs of caudal papillae, subequal spicules, an accessory piece and a prominent anterior lip of the cloaca; female with prominent vulval lips; eggs oval.

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Echinobothrium acanthinophyllum, n.sp. from the spiral valve of *Raja montagui* Fowler

By GWENDOLEN REES, D.Sc.
Department of Zoology, University College of Wales, Aberystwyth

(*Received* 27 *January* 1961)

While examining the spiral valve of *Raja montagui* Fowler at Plymouth in August 1959 Dr H. H. Williams found several specimens of *Echinobothrium* van Ben. which he kindly handed to me. All except one of these were *E. affine*, Diesing. The remaining specimen did not agree with the descriptions of any of the eight existing species but has some distinctive features which indicate that it is new. A search for more specimens has been made in the preserved spiral valve of the fish from which it was obtained but without success. Other fish will be examined as opportunity arises, but in the meantime a brief description of the diagnostic features of this form is given below. It is compared with the known species of the genus.

The scolex is very characteristic and essentially similar in all species of the genus *Echinobothrium*. A detailed description of the functional morphology of the scolex of *E. brachysoma*, Pintner has already been made (Rees, 1961). The genitalia, also, are very similar in general arrangement throughout the genus. They have been described for *E. brachysoma* and *E. affine* and are of the same general pattern in the present case.

The specimen was slightly flattened at the time of collecting, fixed in Duboscq-Brasil and stained with Gower's carmine. The measurements made from this slightly flattened specimen will not be accurate but will give some idea of the size and relative proportions of the various parts. The hooks are unaltered by slight pressure so their measurements will be exact.

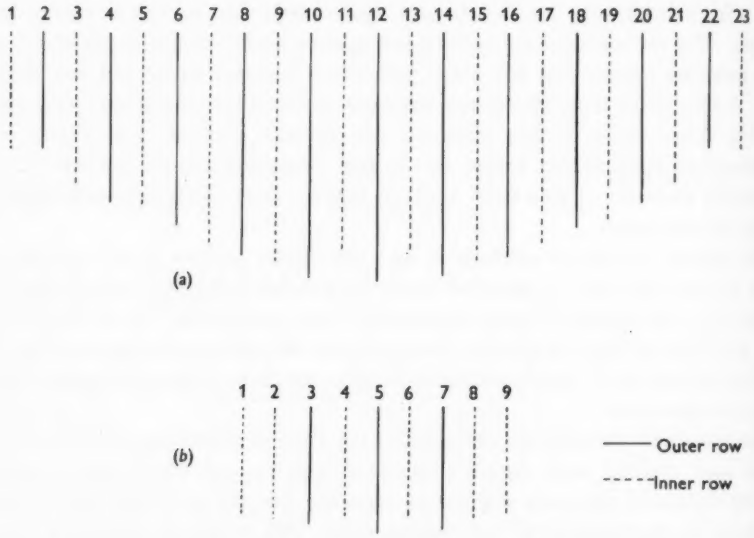
MORPHOLOGY

External features. The worm consists of a scolex and a strobila of seven proglottides, the last, only, being gravid (Pl. 2, fig. 1). It measures 4·22 mm. in length and has a maximum breadth of 0·83 mm. in the gravid proglottid.

The scolex, 0·50 mm. in length, consists of a head proper and a cephalic peduncle (Pl. 1, fig. 1), both of which measure 0·25 mm. in length. The head proper, in spite of being slightly flattened by pressure, seems to be relatively shorter and broader than in other species. It bears a dorsal and a ventral bothridium, 0·40 mm. in length. The bothridia are more foliaceous than in other species and are probably more mobile. They are attached along the whole length of the head proper but being longer, they project backwards over the cephalic peduncle dorsally and ventrally to about half way along its length. The bothridia are armed over the

whole of the outer surface with small backwardly directed spines measuring 0·010 mm. in length anteriorly and posteriorly and 0·011 mm. in the middle region. They taper to a sharp slightly curved point. The face of each bothridium is covered with very tiny closely placed hair-like spines.

Embedded in the anterior end of the head proper is the relatively large rostellum which measures 0·147 mm. in length and has a dorso-ventral diameter of 0·261 mm. Just anterior to the rostellum is a dorsal and a ventral group of apical hooks lying in an arc immediately in front of each bothridium (Pl. 1, figs. 1, 3). Each group consists of 23 large hooks flanked by four small hooks on either side. The large hooks are arranged in two close alternating rows, 11 in the outer and 12 in the inner row. The shape of the hooks is similar to that in other species of the



Text-fig. 1. Diagram, to scale, to illustrate the length of the large hooks in one apical group. (*a*) *Echinobothrium acanthinophyllum* (23) and (*b*) *E. brachysoma* (9).

genus. Of the large hooks 1–4 (and 20–23) are small. There is then a gradual increase in length towards the centre of the row, hook 12 being the longest. The next longest are 10 and 14. Hooks 11 and 13 are shorter than either of these three, being shorter even than 8 and 9 (and 15 and 16). From a plan of the hooks (Text-fig. 1) it can be seen that those of the outer row increase regularly in length towards the centre, but in the inner row there is a steady increase in length of the first five on either side, then the two central ones are shorter. In order of decreasing length therefore for half of a combined row one could arrange the hooks as follows: 12, 10, 9, 8, 11, 7, 6, 5, 4, 3, 2, and 1. In comparing this with *Echinobothrium brachysoma*, which has only 9 hooks, the general arrangement is the same, namely, 5, 3, 2, 4, 1. There is in both cases a shorter hook, which is out of series, on either side of the central or longest hook. The measurements of the hooks in *E. acanthino-*

phyllum are as follows: 0·070 mm. (1 and 23); 0·070 mm. (2 and 22); 0·090 mm. (3 and 21); 0·100 mm. (4 and 20); 0·111 mm. (5 and 19); 0·116 mm. (6 and 18); 0·125 mm. (7 and 17); 0·133 mm. (8 and 16); 0·135 mm. (9 and 15); 0·146 mm. (10 and 14); 0·130 mm. (11 and 13); 0·149 mm. (12). The four small hooks on either side of each main row decrease in length towards the outside, measuring, respectively, 0·041, 0·037, 0·030 and 0·020 mm. Following the head proper is the cephalic peduncle (Pl. 1, fig. 1), 0·25 mm. long and 0·12 mm. broad. It bears 8 longitudinal rows, each of 13 backwardly directed spines. They have the typical shape of the peduncle hooks of the genus, being T-shaped with an inwardly directed root portion arising where the wings join the stem of the T. They decrease in size posteriorly, the anterior hooks measuring 0·076 mm. in length and 0·060 mm. across the wings, the posterior 0·030 mm. in length and 0·030 mm. across the wings. The blade is slightly convex on the outer side and tapers to a point.

The strobila measures 3·72 mm. in length and has a maximum breadth of 0·83 mm. in the gravid proglottid. There is no neck. There are seven proglottides (Pl. 2, fig. 1), the measurements of which are as follows: (1) 0·07 × 0·12 mm., (2) 0·12 × 0·17 mm., (3) 0·11 × 0·22 mm., (4) 0·24 × 0·29 mm., (5) 0·60 × 0·35 mm. (6) 0·84 × 0·42 mm., (7) 1·74 × 0·83 mm. The first three are broader than long, the fourth almost square and the fifth to the seventh about twice as long as broad. The genital atrium is apparent only in the last two proglottides. It is situated on the ventral side in the middle line about one fifth of the length from the posterior margin. There is no uterine pore.

The cuticle is smooth, 0·002 mm. thick and continuous without relation to segmentation.

Musculature. It was not possible, without sections, to study the entire musculature, but much can be seen in the whole mount. The scolex is so similar to that of *Echinobothrium brachysoma* that one would expect the musculature to be of the same general plan.

(*a*) *Intrinsic muscles of scolex.* The superficial musculature of the scolex is only visible at the apex where the superficial longitudinal muscles can be seen crossing over from the left to the right side. They are in the form of scattered fibres fairly regularly arranged in the area between the dorsal and ventral groups of apical hooks (Pl. 1, fig. 4) on the outer side and at right angles to the anterior dorso-ventral muscles which are present in this region.

The dorso-ventral muscles of the head proper are visible behind the rostellum and can be seen extending into the bothridia dorsally and ventrally.

The lateral diagonal muscles, described for *E. brachysoma*, are again fine regularly arranged fibres extending into the bothridia.

The anterior dorso-ventral muscles (Pl. 1, fig. 4) are very clear, passing across the apex of the scolex from near the roots of the dorsal to those of the ventral apical hooks. They are more numerous than in *E. brachysoma*, possibly because the head is larger and the hook row wider. They are delicate fibres parallel to one another and more closely arranged laterally than in the centre. Their contraction will bring the roots of the dorsal and ventral groups of hooks closer together and 'close up' the scolex in front of the withdrawn rostellum.

(*b*) *Rostellum*. The rostellum is embedded in the anterior half of the head proper. Viewed laterally it is rounded behind and flattened or cushion-like in front with a rounded dorsal and ventral lobe below the roots of the apical hooks (Pl. 1, figs. 1–4). It is, therefore, of the same general shape as that of *E. brachysoma*. It measures 0·147 mm. in length and has a maximum dorso-ventral diameter of 0·261 mm. The main mass of the rostellum consists of regularly arranged transverse fibres running from left to right (Pl. 1, fig. 2), the contraction of which will increase the dorso-ventral diameter. As in *E. brachysoma* there is a small tendon projecting into the rostellum posteriorly and the meridional fibres can be recognized passing up the sides of the rostellum, over the top and down the other side. These cross below the anterior dorso-ventral muscles and some are continuous, posteriorly, with the tendon. Contraction of the muscles of the rostellum will compress it laterally and force it to elongate in a dorso-ventral direction. Around the dorsal and ventral lobes of the rostellum are circular muscles (Pl. 1, fig. 4), the contraction of which would render the lobes firmer when they would form a better support for the roots of the elevated apical hooks.

(*c*) *Muscles of the apical hooks*. The movements of the apical hooks are undoubtedly very similar to those described for *E. brachysoma* and will depend partly on muscles which are directly attached to them and partly on the movements of the rostellum. The inner hook elevator muscles can be seen as a band of fibres passing dorso-ventrally around the posterior border of the rostellum (Pl. 1, fig. 2). They curve round the anterior margin dorsally and ventrally and spread out towards the roots of the hooks to which they are attached. In the mid line, posteriorly, is a short tendon with which the fibres of the dorsal and ventral sides are continuous. Contraction of these muscles will raise the blades of the hooks and help to push the rostellum forwards. The outer hook elevator muscles described for *E. brachysoma and E. affine* could not be seen but may be present.

(*d*) *Extrinsic muscles of rostellum and hook apparatus*. The rostellum is provided with a dorsal and a ventral protractor muscle (Pl. 1, fig. 2). Each consists of a bundle of fibres and is shorter and thicker than in *E. brachysoma* and *E. affine*; they occupy the relatively small space between the rostellum and the cephalic peduncle. Where the peduncle joins the head proper the fibres spread out and are inserted into the basement membrane dorso-laterally and ventro-laterally on either side. Anteriorly each muscle narrows and becomes continuous with a tendon which passes around the outer side of the hook elevator muscles to be inserted into the basement membrane just behind the hook row. The contraction of these muscles will help in the protrusion of the rostellum.

The movement of the whole apparatus of rostellum and hooks is brought about by the concerted action of the intrinsic muscles of the rostellum, the protractors, the hook elevator muscles and the muscles at the apex of the scolex.

The hooks on the cephalic peduncle were all in the resting position (Pl. 1, fig. 1), so that their elevator muscles could not be seen.

Genitalia. The general arrangement of the genitalia seems to be fairly constant throughout the genus, the course of the reproductive ducts varying slightly according to the position of the genital atrium. In *Echinobothrium acanthino-*

phyllum the genital atrium is in much the same position as in *E. brachysoma* near the posterior end of the proglottid (Pl. 2, fig. 1); it is apparent only in the last two proglottides. In the last, the only gravid proglottid, it is a transversely oval aperture 0·054 × 0·004 mm. situated 0·33 mm. from the posterior margin. In the mature proglottid next in front it is rounded 0·027 × 0·027 mm. and 0·818 mm. from the posterior margin. The cirrus and vagina open side by side into the genital atrium.

(*a*) *Male reproductive system.* There are 13 testes in proglottides 3–6. They are not recognizable in proglottides 1 and 2 and are obscured by the uterus or have degenerated in proglottid 7. In proglottid 3 they are closely packed in a double row with one median testis in front and are transversely oval, measuring 0·031 × 0·011 mm. In proglottid 4 they are larger and have become wedge shaped, the narrow end being towards the centre. They are arranged in the same way and measure 0·056 × 0·025 mm.; the anterior testis is diamond-shaped and measures 0·061 × 0·022 mm. In proglottid 5 they have increased to 0·090 × 0·034 mm. and the anterior testis to 0·072 × 0·025 mm. In proglottid 6, which is longer, they are not so closely packed and become oval in outline, measuring 0·102 × 0·052 mm. The cirrus sac is small, pyriform, 0·118 × 0·090 mm. and directed posteriorly. The cirrus seems to be relatively short. In proglottid 6 a small portion of it was evaginated. It is covered with short straight spines 0·004 mm. long. The remainder of the armed cirrus could be followed into the cirrus sac where it forms a small loop and becomes continuous with the vas deferens.

(*b*) *Female reproductive system.* The ovary is bilobed and posterior the two lobes being united by an isthmus from the posterior side of which arises the ovicapt or beginning of the oviduct. The course of the vagina could not be followed along its whole length, so it is not known whether it loops around the cirrus sac as in some species. Just in front of the isthmus the vagina enlarges to form a receptaculum seminis which narrows as it passes backwards beyond the isthmus to open into the oviduct. Mehlis's gland lies just behind in the middle line between the lobes of the ovary. The uterine duct emerges posteriorly, turns forwards dorso-laterally, runs up the proglottid to about half way and opens into the dorsal side of the sac shaped uterus. This arrangement is very similar to that described for *E. brachysoma*. The uterine duct throughout its length contains eggs. Immediately beyond Mehlis's gland they are oval, consisting of an oocyte and two yolk cells one behind the other. Passing along the uterine duct the eggs become rounded, but the capsules are not fully formed. From the uterine duct the eggs, now measuring 0·013 × 0·013 to 0·015 × 0·011 mm., pass in groups into the uterus. They are, at first, in loose somewhat irregular clusters of 8–10, but after a time these clusters 'tighten', the eggs being pressed closely together so that their adjacent surfaces become flattened (Pl. 2, figs. 1, 2). The clusters are eventually almost spherical, measuring 0·050 × 0·040 to 0·055 × 0·043 mm. The capsules are yellowish brown and without appendages and are not fully formed until the eggs are compressed into clusters. The large transversely oval yolk follicles are lateral on either side.

THE INTESTINE OF THE HOST

No specimens, unfortunately, have been found attached to the spiral valve of the host. The mode of attachment, however, must be somewhat similar to that described for *Echinobothrium brachysoma* in the spiral valve of *Raja clavata*.

The internal surface of the spiral valve of *Raja montagui* differs from that of *R. clavata*. In the latter the scolices of *E. brachysoma* fit perfectly into the tubular crypts which cover the internal surface of the spiral valve, the elevated hooks piercing the wall and anchoring the worm. In *R. montagui* the surface is covered with closely arranged bluntly rounded folds, 0·75–1·00 mm. in width, some of which are short, some long and sinuous, and others slightly branched (Pl. 2, fig. 3). They form a mosaic-like pattern over the whole surface, and will be referred to as the primary folds. On close examination and in horizontal sections (Pl. 2, figs. 4, 5) smaller vertical folds 0·27–0·037 mm. in depth can be seen placed close together extending down on either side of the primary folds. Adjacent vertical folds may fuse to form tubular cavities open at one or both ends. All the folds are covered with a layer of epithelial cells similar to those lining the crypts in *R. clavata*. At first sight this does not appear to be such an ideal surface, as that of the spiral valve of *R. clavata*, for the attachment of a scolex of *Echinobothrium*. The scolex of *E. acanthinophyllum* is, however, much larger than that of *E. brachysoma*. It could not, in fact, fit into the crypts in the spiral valve of *Raja clavata* which may be one of the factors involved in the choice of host. The bothridia, also, are larger, more foliaceous and armed with spines over the outer surface and the apical hooks are longer and more numerous. The structure of the spiral valve of *R. montagui* is probably much better suited to a scolex of these proportions. If it is thrust between the primary folds of the spiral valve it could be manoeuvred by the mobile armed bothridia into a suitable channel between the primary or vertical folds, the hooks elevated and the scolex anchored to the epithelium. Provided there was a surface opposite the dorsal and ventral apical hooks the fact that the 'tube' in which the scolex was contained was incomplete opposite one or even both lateral surfaces would be immaterial.

The peduncle hooks undoubtedly assist at times in anchoring the scolex; the strobila, presumably, would be free in the lumen of the spiral valve.

DISCUSSION

Eight species of the genus *Echinobothrium* have already been described. They are *E. typus*, van Beneden (1849); *E. affine*, Diesing (1863); *E. musteli*, Pintner (1889); *E. brachysoma*, Pintner (1889); *E. rhinoptera*, Shipley & Hornell (1906); *E. longicolle*, Southwell (1925); *E. rayii*, Heller (1949) and *E. Mathiasi*, Euzet (1951). In addition, two larval forms are known, namely, *E. levicolle*, Lespès (1857) and *E. Benedeni*, Ruszkowski (1927).

The present species can be distinguished from *E. rhinoptera* as the whole of the cephalic peduncle is armed with spines and from *E. longicolle* and *E. Mathiasi* in having less than 50 spines in each row on the peduncle. It differs from *E. musteli* in having no spines on the rostrum, in the number of apical hooks and in other

features. *E. typus*, *E. brachysoma* and *E. affine* can be distinguished from the present form by the number of apical hooks, the number of spines in each row on the peduncle, details of the genitalia and the shape and arrangement of the eggs.

Of the larval forms *E. levicolle* is described as having more than 20 hooks on each side, but there is little information about it. Southwell (1925) stated that 'this larva cannot rank as a species'. *E. Benedeni*, described by Ruszkowski (1927) from *Hippolyte varians* Leach has 26 long apical hooks in each group flanked by 4 small hooks. This is three more than in the present form, but the same number as in *E. Mathiasi*. Euzet (1951) stated that even though the number of apical hooks is the same in these two species, the two cannot be associated with one another as the peduncle hooks are not developed in *E. Benedeni* and little is known of the rest of the anatomy except that it has 9 or 10 testes per segment.

Echinobothrium acanthinophyllum sp.nov. is, therefore, distinct from previously known species and can be diagnosed as follows:

Diagnosis. Body consists of a scolex and seven proglottides, the last being gravid; scolex bears two foliaceous bothridia armed with short spines over the outer surface; 23 large apical hooks in each group flanked by 4 small hooks on either side; large hooks measure 0·070–0·149 mm. in length, small hooks 0·020–0·041 mm.; cephalic peduncle bears 8 longitudinal rows of 13 spines measuring 0·076 × 0·06 mm. anteriorly and 0·03 × 0·03 mm. posteriorly; last proglottid about twice as long as broad; genital atrium mid-ventral one fifth of the length from the posterior margin; 13 testes; cirrus sac pyriform, small; eggs 0·013 × 0·013 to 0·015 × 0·011 mm. on entering uterus, join to form clusters of 8–10, the clusters measuring 0·050 × 0·040 to 0·005 × 0·043 mm.; capsules yellow without appendages. In spiral valve of *Raja montagui*, Fowler.

Euzet (1951) compiled a key for the identification of 7 species of *Echinobothrium* van Ben. I give below a modification of this key to include *E. rajii* Heller, which was omitted and *E. acanthinophyllum* sp.nov. As the number of testes may be variable within a species and even from one proglottid to another in the same strobila, this character alone is not considered to be sufficient to separate species.

Modifications of Euzet's (1951) key to the species of *Echinobothrium*:

1. Part only of the cephalic penduncle armed with spines	*E. rhinoptera*
Cephalic penduncle armed along whole length	2
2. More than 100 spines in each row on peduncle	*E. longicolle*
50–60 spines in each row on peduncle	*E. Mathiasi*
Less than 50 spines in each row on peduncle	3
3. Rostrum armed with small spines in addition to crown of hooks	*E. musteli*
Rostrum not armed with small spines	4
4. Less than 20 large hooks in each apical group 7 large hooks, 12–13 spines in each row on peduncle, 8–10 testes	*E. typus*
9 large hooks, 14–16 spines in each row on peduncle, 6–9 testes	*E. brachysoma*
11 large hooks, 26–28 spines in each row on peduncle, 8–14 testes	*E. affine*
More than 20 large hooks in each apical group	5
5. 23 large hooks, 13 spines in each row on peduncle, 13 testes	*E. acanthinophyllum*
26 large hooks, 8 spines in each row on peduncle, 16 testes	*E. rajii*

The two larvae are not included in the key.

SUMMARY

1. *Echinobothrium acanthinophyllum* sp.nov. occurs in the spiral valve of *Raja montagui* Fowler. *E. affine* Diesing was also present.

2. *E. acanthinophyllum* possesses 23 large hooks in each apical group flanked by 4 small hooks on either side. There are 13 spines in each of the 8 longitudinal rows on the cephalic peduncle and 13 testes in each proglottid. The ripe eggs aggregate into clusters of 8–10, which fill the gravid uterus.

3. Euzet's key is revised to include the 9 species of the genus.

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EXPLANATION OF PLATES

(Photomicrographs by D. Hemingway Jones, M.Sc., to whom I am most grateful.)

PLATE 1. *Echinobothrium acanthinophyllum* sp.nov.

Fig. 1. Scolex, lateral view, focused to show apical hooks and peduncle hooks.

Fig. 2. Scolex, lateral view, focused to show the spines on the bothridia, the intrinsic muscles of the rostellum, the two protractors and the elevator muscles of the apical hooks.

Fig. 3. The two apical groups of hooks. Of the 23 large hooks there is an overlap in two places on either side at the far end of each row (hooks 2 and 3, 4 and 5). All four small hooks at the ends of each row are not in focus.

Fig. 4. Apex of scolex showing superficial longitudinal muscles, anterior dorso-ventral muscles and the circular muscles around the dorsal and ventral lobes of the rostellum.

PLATE 2. *Echinobothrium acanthinophyllum* sp.nov.

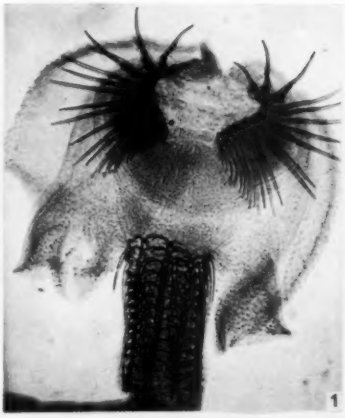
Fig. 1. Entire, slightly flattened specimen. Last proglottid, only, gravid.

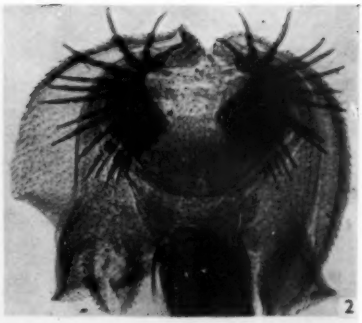
Fig. 2. Egg clusters in uterus of gravid proglottid.

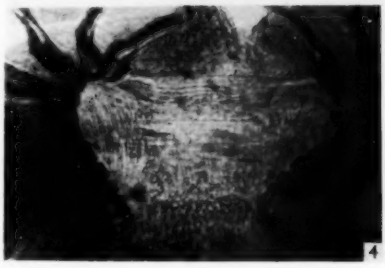
Fig. 3. Internal surface of spiral valve of *Raja montagui* Fowler showing primary and vertical folds.

Fig. 4. Horizontal section through primary folds showing vertical folds on either side, some joining to form tubes.

Fig. 5. An enlarged portion of the same.







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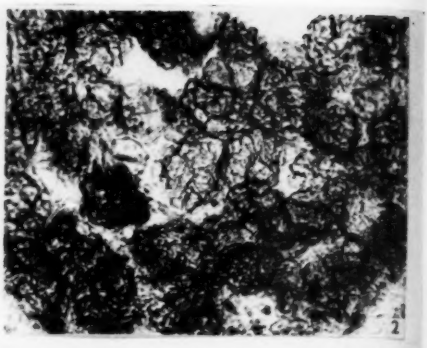

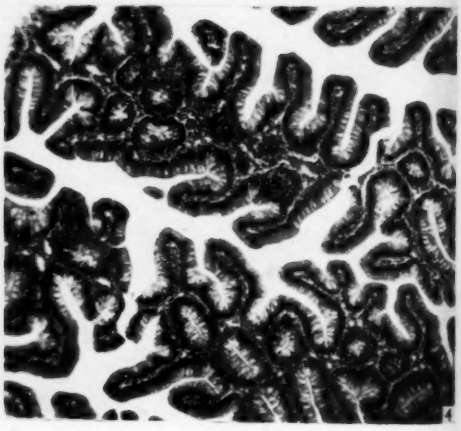

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The protozoa occurring in the hind-gut of cockroaches

I. Responses to changes in environment

By H. M. D. HOYTE

*Molteno Institute, University of Cambridge**

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I. GENERAL INTRODUCTION

(*a*) *Historical survey*

Protozoa in cockroaches were first described by von Siebold (1839). This paper also seems to be the first record of Protozoa inhabiting the lumen of the hind-gut of an orthopteran, as opposed to invading the tissues of the host. The two types he described were (1) a *Leucophrys*-like infusorian and (2) a *Proteus*-like animal, both in *Blatta orientalis*. Semans (1943*b*) thought the ciliate was *Nyctotherus ovalis*. Certainly the shape of *Leucophrys* is like that of *Nyctotherus*, but the cytostome is more like that of *Isotricha caulleryi*; the identity of the ciliate must remain uncertain. The description of the *Proteus*-like animal (overlooked by Semans) strongly suggests that von Siebold saw *Endamoeba blattae*. Stein (1867) recognized the same organism in the globose 'Amöbenform' which he found in large numbers in a single specimen of *Blatta orientalis*. The observations of these

* Present address: University of Queensland Veterinary School, Brisbane, Australia.

two authors were extended by Bütschli (1878), who named the species *Amoeba blattae*. This was later changed to *Endamoeba blattae* by Leidy (1879).

Nyctotherus ovalis was first described by Leidy (1850), from the hind-gut of *Blatta orientalis*. Stein (1854) called it *Bursaria blattarum* when he found it in

Table 1. *Records of Protozoa in the hind-gut of cockroaches*

Protozoa	Cockroaches				
	Blatta orientalis	*Periplaneta americana*	*Periplaneta australasiae*	*Blattella germanica*	*Blaberus giganteus*
Class *ZOOFLAGELLATA*					
Order TRYPANOSOMIDEA					
Leptomonas periplanetae	Laveran & Franchini (1920)	—	—	—	—
Order TRICHOMONADINA					
Trimitus blattae	Grassé (1952)	—	—	—	—
Monocercomonas orthopterorum	Parisi (1910), Hoyte	—	—	—	—
Tetratricho-mastix blattidarum	Young (1935)	Young (1935)	—	Young (1935)	—
Monocercomon-oides orthopterorum	Bŏlař (1916), Hoyte	Hoyte	—	—	Hoyte
Order RETORTAMONADINA					
Retortamonas blattae	Bishop (1931)	—	—	—	—
Order LOPHOMONADINA					
Lophomonas blattarum	Stein (1860), Hoyte	Schuster (1898), Hoyte	—	(G)	Hoyte
L. striata	Bütschli (1878), Hoyte	Schuster (1898) Hoyte	—	(G)	—
Order DISTOMATA					
Hexamita peri-planetae	Bŏlař (1916)	—	—	—	—
Class *LOBOSA*					
Order AMOEBAEA					
Hartmanella blattae	Ivanić (1937)	—	—	—	—
Endamoeba blattae	von Siebold (1839), Hoyte	Lucas (1928), Hoyte	Meglitsch (1940)	—	Hoyte
Entamoeba thomsoni	Lucas (1927), Hoyte	Lucas (1927), Hoyte	—	McAdow (1931)	—
E. hertwigi	Epstein (1941)	—	—	—	—
E. dofleini	Epstein (1941)	—	—	—	—
Endolimax blattae	Lucas (1927)	Lucas (1927)	(S)	—	—
Class *CILIATA*					
Order HOLOTRICHA					
Isotricha caulleryi	—	Weill (1929)	—	—	—
Order SPIROTRICHA					
Balantidium blattarum	—	Ghosh (1922*a*)	—	—	—
B. ovatum	—	Ghosh (1922*b*)	—	—	—
B. praenucleatum	Kudo & Meglitsch (1938)	—	—	—	—
Nyctotherus ovalis	Leidy (1850), Hoyte	Schuster (1898), Hoyte	(S)	Stein (1854), Hoyte	Hoyte

Blatta orientalis and *Blattella germanica*: he seems to be the first author to have extended his study of Protozoa in cockroaches to species other than *Blatta orientalis*.

The names of the Protozoa which have been recorded in the hind-gut of cockroaches are listed in Table 1. The cockroaches which I have been able to study are the five species: *Blatta orientalis*, *Periplaneta americana*, *P. australasiae*, *Blattella germanica* and *Blaberus giganteus*. Opposite the name of each protozoan is noted the name of the author (and the date) who first gave a recognizable description of the species, but did not necessarily name it. The names of the authors are, in turn, listed under the name of the cockroach in which the parasite was found. Thus, *Endamoeba blattae* was first seen in *Blatta orientalis* by von Siebold (1839), in *Periplaneta americana* by Lucas (1928), and in *P. australasiae* by Meglitsch (1940).

The classification of the Protozoa (excluding the Ciliata) is that adopted by Grassé (1952, 1953): the classification of the Ciliata is that used by Kudo (1946). The classification of the cockroaches is that used by Hincks (1949).

Of great assistance in compling this list have been the two papers (1943*a*, *b*) by Semans. They also include copious references on Protozoa parasitic in Orthoptera other than these five species of cockroaches, and on the Sporozoa—which are outside the scope of this study.

The letters (G) and (S), which recur in the table, singify that Grassé (1952, 1953) or Steinhaus (1946), respectively, mentioned that the particular protozoan occurs in that species of cockroach; but that I have been unable to find the original reference.

Many of these Protozoa occurred in my stocks of cockroaches: they are noted as 'Hoyte'.

The following are notes on some of the individual records:

Leptomonas periplanetae was described under the name of *Herpetomonas periplanetae*.

Tejera (1926) described *Leptomonas blaberae* in *Blabera* [= *Blaberus*] sp.

Monocercomonas orthopterorum was described under the name of *Trichomonas* (*Trichomastix*) *orthopterorum* by Parisi (1910). This name was emended by Bělař (1916) to the present one; but unfortunately his cockroaches seem to have harboured not one but two species of flagellate: *Monocercomonas orthopterorum*, and the species now named *Monocercomonoides orthopterorum*. The latter he regarded as a division stage of the former. The latter had not been seen by Parisi and therefore Bělař's is the first record of it. The genus *Monocercomonoides* (= *Monocercomonas* Grassi) was erected by Travis (1932).

Semens (1943*a*) followed Wenyon (1926) in thinking that *Monocercomonas orthopterorum* should be called *Retortamonas orthopterorum*, (and overlooked *Monocercomonoides*). However, Wenrich (1932) considered that the species of *Embadomonas* MacKinnon are co-generic with the older *Retortamonas gryllotalpa*, and approved the retention of the genus *Monocercomonas*. Certainly, *Monocercomonas orthopterorum* is not co-generic with the *Embadomonas–Retortamonas* type.

Tetratrichomastix was considered by Grassé (1952) to be a subgenus of *Monocercomonas*.

Retortamonas blattae was described under the name of *Embadomonas blattae*.

Lophomonas striata in *Periplaneta americana* was described under the name of *L. sulcata*. Schuster (1898) considered that the characters of the *Lophomonas* he discovered differed sufficiently from those described for *L. striata* to warrant the erection of a new species. However, Kudo (1926) pointed out that the characters of *L. striata* do comprehend those differences attributed to *L. sulcata*.

Hexamita periplanetae was described under the name of *Octomitus periplanetae*.

Endamoeba blattae: the history of the discovery of this organism is outlined on a previous page.

McAdow (1931): I have not seen this paper. Semens (1943*b*) refers to it as an M.A. Thesis: its contents seem to be unpublished.

(*b*) *Material*

The species of cockroaches and the Protozoa which they harboured are named in the previous section. Some further details must be given. The *Blatta orientalis* had been caught in Cambridge, but from two different localities. The first stock harboured *Monocercomonoides orthopterorum*, *Lophomonas blattarum*, *L. striata*, *Endamoeba blattae* and *Nyctotherus ovalis*. (Individual specimens rarely harboured all these species: *L. blattarum* occurred least frequently; *Monocercomonoides* and *Nyctotherus* occurred in large numbers in most.) The second stock of *Blatta* harboured, in addition to the above, *Monocercomonas orthopterorum* and *Entamoeba thomsoni*. These infected a very low percentage of the specimens, and because this second stock was established long after the first had built up, little work could be done on these two species.

The *Periplaneta americana* were also kept in two stocks. One originated from the Zoological Gardens in London, and the other was compounded of cockroaches from Edinburgh Zoological Gardens, from the Department of Zoology in the University of Cambridge and from another Cambridge locality. Both stocks harboured the same species of Protozoa, and both were used.

The *P. australasiae* originated from Ceylon, and were few in number. Only one survived for any length of time: both it and the ones that died seemed to be free of Protozoa.

The *Blattella germanica* consisted of two stocks, both heavily infected with *Nyctotherus ovalis*. The *Blaberus giganteus* were kept as a temporary small stock, which was replenished from those maintained in the Cambridge Department of Zoology as the specimens were used in experiments.

Thus, the five species of cockroaches harboured between them only seven out of the twenty recorded species of Protozoa. The greatest variety occurred in *Blatta orientalis* and *Periplaneta americana*. The second point of interest is that, although the historical survey suggests that a greater variety of flagellates occur in *Blatta* and a greater variety of ciliates in *Periplaneta*, there was little sign of these habitat preferences in these stocks.

(*c*) *Methods*

The stocks of cockroaches were maintained at a temperature of about 25 °C. The food given to *Periplaneta* and *Blatta* was basically rat-cake, horse-heart meat and apple; lettuce was also given to one of each pair of stocks. *Blattella* received horse-heart meat and apple, whereas *Blaberus* received lettuce and bread. No drinking water was given.

Some *Periplaneta* and *Blatta* were reared free of Protozoa and used in a variety of experiments on the means of initiating infection. The method was that found successful by Wollman (1926), who reared generations of aseptic *Blattella*. Egg-cases were collected from the tanks and washed three times in 1 in 2,000 parts of mercuric chloride in water for a total time of 4–5 min. They were then rinsed thoroughly in distilled water and kept in sterile flasks or test-tubes plugged with cotton-wool. About a quarter of the egg-cases hatched during the following few weeks. The others had failed in various stages of development. The proportion which hatched could not be improved by keeping the egg-cases in a moist environment or even by not treating them with mercuric chloride.

(*d*) *Aims*

The life-cycle of each of these Protozoa seems to consist of a simple alternation between trophozoite and cyst. The relationship between host and parasite and the mode of transmission also seem to be simple. The aims of these studies were to examine some aspects of the host parasite relationship: especially the effects of some changes in the environment of the host on the parasite, of changes within the host on the parasite and modifications of *Nyctotherus ovalis* related to the species of host. The second aim was to study the structure and the biology of the encysted *Nyctotherus*, in particular its viability at different temperatures and at extremes of environmental humidity. The third feature of the life-cycle of *Nyctotherus* to be examined was the mode of transmission, and the factors determining excystation and the establishment of infection in a new host.

The description of the work falls conveniently into three parts, the first of which is presented here.

II. THE PROTOZOA IN RELATION TO THE ENVIRONMENT INSIDE THE COCKROACH

(*a*) *Influence of variations in humidity and temperature of the host's environment upon the Protozoa*

Material. Large nymphal *Periplaneta americana* from a stock heavily infected with *Nyctotherus*, *Endamoeba*, *Lophomonas striata* and *Monocercomonoides*; also, adult and large nymphal *Blatta orientalis* from stock heavily infected with the first three types of Protozoa.

Methods. Six *Periplaneta* were put in each of four boiling-tubes, fitted with muslin covers. Two tubes were put in a desiccator (calcium chloride flakes: 0% relative humidity), and two in a 'desiccator' with water in the bottom (100%

relative humidity). All were placed in a constant-temperature room at 25° C. Once a day, the lids were removed from the desiccators for about a minute, to allow the air within to equilibrate with that of the open air.

The cockroaches in one tube of each pair were killed at the end of one week and the contents of their hind-guts examined. The cockroaches in the second tubes were examined at the end of two weeks.

The experiment was repeated with *Blatta*.

Results. At the end of 1 week, the numbers of Protozoa in the *Periplaneta* kept at 100% relative humidity appeared to be unaffected; but in those at 0% the numbers of all four types of Protozoa had declined. At the end of 2 weeks, those *Periplaneta* in the water-saturated atmosphere still supported a heavy fauna of all four types of Protozoa. However, *Endamoeba*, *Lophomonas* and *Monocercomonoides* had disappeared from those in the dry atmosphere; and *Nyctotherus* was present in only small numbers in some hosts and had disappeared from the others. One cockroach had died in each of the pair of tubes at 0% relative humidity.

Similar results were got with *Blatta*. At the end of 1 week, *Nyctotherus*, *Endamoeba* and *Lophomonas* were present in apparently unaltered numbers in those cockroaches kept at 100% relative humidity; but in those at 0% only *Lophomonas* showed any decline. However, at the end of 2 weeks, all six cockroaches were dead. Two had evidently died just before the end of the experiment and were found to be still supporting a small number of *Nyctotherus*, but no *Endamoeba* or *Lophomonas*.

Material

Adult and large nymphal *Blatta orientalis* from a stock heavily infected with *Nyctotherus*, *Endamoeba* and *Lophomonas striata*.

Series I

Methods. Four groups of six cockroaches were kept at 37 °C., in a water-saturated atmosphere. One group of six cockroaches was inspected after 1, 2, 3 and 7 days of treatment.

Results. After 1 day, all six cockroaches were alive and moving actively, and all three types of Protozoa survived in good numbers. However, after 2, 3, and 7 days, two or three cockroaches in each pot had died. The remainder were mostly moribund and sluggish, but at least one fully active cockroach survived in each pot.

Trophozoites of *Nyctotherus* survived in some cockroaches for 2 and 3 day but by the latter time were patently moribund. The proportion of degenerate cysts was small on the second day, but rapidly increased at the same time as the absolute numbers fell off.

No trophozoites of *Endamoeba* were seen after the first day, except that one cockroach harboured a few for the full 7 days, and these appeared to be quite normal. Cysts were also scarce after the first day.

Lophomonas striata survived in small numbers up to only the second day. Cysts were not searched for. *Monocercomonoides* was seen once, in small numbers,

in one of the hosts examined after 2 days. Infection with this flagellate was not widespread in this stock of *Blatta*, hence nothing can be deduced from its absence in all of the other experimental animals.

Series II

Methods. Two groups of six *Blatta* were put in a desiccator, the bottom of which was covered with calcium chloride flakes, and kept at 37° C.

Results. After 1 day, three of the cockroaches in the first pot were alive and active, one was dead and two were moribund. *Nyctotherus*, *Endamoeba*, *Lophomonas striata* and *Monocercomonoides* survived in good numbers among the first three hosts, but were disappearing from the others. After 2 days, all cockroaches in the second pot were dead and their bodies dried out.

Series III

A few of the cockroaches survived at 37 °C. in an atmosphere of 100 % relative humidity for 7 days, but all were killed at 0 % in less than 2 days. In this series, relative humidities of 80 and 90 % were tried, as being probably more suitable for the cockroach than a fully saturated atmosphere.

Methods. Buxton (1931) described several methods of maintaining atmospheres of various relative humidities. Using this data, suitable solutions of sulphuric acid, and of potassium hydroxide, were made up and substituted for the water in the bottom of the 'desiccator'. Sulphuric acid was used in the first two experiments to maintain an atmosphere of 80 % relative humidity, but, because it was suspected that fumes from the acid solution were killing the cockroaches, potassium hydroxide solutions were used to maintain humidities of 80 and of 90 %.

Each experiment was run for 2 days. In other respects, the same procedure was used as in the previous experiments.

Results. Nearly all of the cockroaches in all four experiments were killed by the treatments, and it was noted that there was no apparent difference between the sexes in this respect. The best result was actually obtained in one of the experiments using the sulphuric acid solution. Here, trophozoites of *Nyctotherus* survived in fair numbers in the one cockroach which remained alive. Otherwise, all that survived were cysts, many of which were degenerate, and cysts of *Endamoeba* and *Lophomonas striata*.

Conclusions. These results indicated that at 25° C., both *Periplaneta* and *Blatta* survive in a water-saturated atmosphere better than they do in a completely dry one. Likewise, the Protozoa in their hind-guts are able to flourish in the former environment, but not in the latter. This suggests that their well-being is linked with that of their host.

A few of the *Blatta* kept at 37 °C. in an atmosphere of 100 % relative humidity were able to survive for 7 days. At 100 % relative humidity, there can be no cooling of the body by water loss; and Necheles (1924) showed that under these conditions, the body-temperature of *Periplaneta* is slightly higher than that of the environment.

The earlier death of *Blatta* kept at 37° C. and 0% relative humidity confirmed the results of Gunn (1935).

Since the Protozoa in those at 100% relative humidity also survived, though in declining numbers, it can be concluded that a temperature of 37° C. is not itself immediately lethal to the Protozoa. For if it is, all would presumably have died within 24 hours, as did the Protozoa of the termite studied by Cleveland (1924).

(*b*) *Influence of increased oxygen pressure in the host's environment upon the Protozoa*

Introduction

Cleveland (1925*a*) described how the termites of the genus *Termopsis* can be selectively defaunated by maintaining them in pure oxygen at 1 to 1½ atmospheres pressure for various periods of time. In a later paper (1925*b*), he described how he had used this technique to kill the gut Protozoa in other termites, in *Periplaneta americana*, in earthworms, in *Rana pipiens* adults and tadpoles, in goldfish and in *Necturus* the salamander. He found the technique was inapplicable to rats because the hosts were killed before the Protozoa; and Bishop (1927), working with *Macacus rhesus*, concluded that an increase in oxygen pressure, within the limits tolerated by the monkey, had no real effect in eliminating the intestinal Protozoa.

Cleveland found that a pressure of 3½ atmospheres of oxygen for 3½ hr. would kill all the *Nyctotherus* and *Balantidium* in *Periplaneta*, but that 40 min. treatment was long enough to kill all the *Lophomonas* and *Polymastix*. The method he used in determining the Protozoa in a cockroach was to squeeze its abdomen and examine the sample of gut contents so obtained. However, I have found this method to be unreliable when numbers of Protozoa are small. I therefore repeated his work and extended it to include other species of cockroaches.

Series I

Material. Adults and large nymphal specimens of *Periplaneta americana*, *Blatta orientalis*, *Blaberus giganteus* and *Blattella germanica*. The first two were known to be infected with *Nyctotherus ovalis*, *Endamoeba blattae*, *Lophomonas striata* and *Monocercomonoides orthopterorum*. *Blaberus* and *Blattella* were infected regularly only with *Nyctotherus*. *Endamoeba* and *Lophomonas blattarum* also occurred in *Blaberus*, but too infrequently for proper study.

Methods. Groups of four to six cockroaches of each species were subjected to 3½ atmospheres pressure of oxygen for different periods of time. The cockroaches were contained in glass pots, fitted with wire gauze lids. The main part of the apparatus was the pressure chamber, made of a pressure-cooker bored with two holes to take capillary brass tubing. The inlet was connected, via a valve and pressure gauge, to a cylinder of oxygen under high pressure. The outlet led to a reduction valve.

At the start of each experiment, the lid of the chamber was closed and oxygen swept through for at least 10 min., the reduction valve being wide open. The valve

was then nearly closed and the pressure raised to 2½ atmospheres pressure (37–38 lb. per square inch) above atmospheric pressure and maintained at that until the end of the treatment. The temperature was maintained at 25° C.

By leaving the reduction valve slightly open, it was possible to have a flow of oxygen through the apparatus at a rate of about 2 l. per hour. To offset the dehydrating effect of this dry gas, an inch or so of water was put in the bottom of the pressure chamber before the start of the experiment.

A second modification was found to be necessary. If the gut contents of a cockroach is examined immediately after the end of the treatment, many of the Protozoa are seen to be immobile, and it is difficult to tell if this condition is merely temporary or if they are really dead and will presently decay. Cockroaches were therefore left for 12 hr. or more in air at room temperature before they were dissected.

Results. In any one species of cockroach, the different species of Protozoa survived for different lengths of time. *Nyctotherus* was the most hardy, *Monocercomonoides* the least so. All species of Protozoa in *Blatta* survived for a longer time than in *Periplaneta*, and *Nyctotherus* survived in *Blaberus* and *Blattella* longest of all. The complete results are summarized in Table 2.

Table 2. *Maximum survival times of Protozoa, in hours*

	Periplaneta	*Blatta*	*Blaberus*	*Blattella*
Monocercomonoides orthopterorum	½–½	¾–1	—	—
Lophomonas striata	1–3½	5–7	—	—
Endamoeba blattae	1–3½	7–8	—	—
Nyctotherus ovalis	7–12	16–21	21<	21<

It must be emphasized that the time limits listed in the Tables are all maximum survival times. For instance, some *Nyctotherus* survived in some *Periplaneta* which had been treated for 7 hr., and all *Nyctotherus* in all *Periplaneta* were killed after 12 hr., treatment. Some *Periplaneta*, however, were defaunated in less than 7 hr., and even in those which resisted, some of the *Nyctotherus* were killed. Both *Blaberus* and *Blattella* were killed by 21 hr. of treatment, but *Nyctotherus* still survived in some.

The effect of the treatment on some *Nyctotherus* was to cause the body to round up into a sphere, the cilia to move slowly or to stop beating altogether, and in some, the macronucleus to elongate. This is reminiscent of the condition of *Nyctotherus* in a moulting cockroach (see Part III).

No variation was found in the abilities of the Protozoa to survive in adult males or females or in nymphs. However, the nymphs themselves seemed to survive the treatment better than the adults. As with the Protozoa, the effects of oxygen poisoning in the cockroach seemed to be reversible. (The chief symptoms of oxygen-poisoning were (1) impaired co-ordination of leg movements with balance and (2) decreased spontaneous activity. The severity of these increased with increased time spent in the oxygen-chamber.)

Series II

Material. Three adult and three nymphal *Periplaneta americana*, all heavily infected with *Nyctotherus ovalis* but no other Protozoa.

Methods. The cockroaches were kept in oxygen at atmospheric pressure for 6 days. They were confined in a Dreschel's gas washbottle and given a supply of apple and rat-cake. A gentle flow of oxygen was maintained throughout the 6 days.

Results. *Nyctotherus* survived in good numbers in four of the cockroaches, but had been eliminated from another. The sixth was dead. The *Nyctotherus* were normal in appearance: there were none like the ones noticed in Series I as being reminiscent of the condition in moulting cockroaches.

Series III

The survival time of *Nyctotherus* differed in *Periplaneta*, *Blatta*, *Blaberus* and *Blattella*. This could be due to a genuine variation in the resistance of the different populations of ciliate to oxygen, or it could reside in a difference in the anatomy or physiology of the cockroaches which results in some populations of *Nyctotherus* being more protected than others. To test these two possibilities, cockroaches which had been reared free of Protozoa, were infected with *Nyctotherus* from the same and from other species of cockroach.

Material. (1) Nymphal *Periplaneta*, *Blatta* and *Blaberus*, all free of Protozoa. (2) Heavily infected faeces from *Periplaneta*, *Blatta* and *Blaberus*.

Methods. The initial steps are described fully in the second paper of this series, part of which describes studies on the sizes of the cysts of *Nyctotherus*. Briefly, infections were established in the nymphs by feeding them on two occasions with faeces containing *Nyctotherus* cysts. Each nymph received faeces from the same source on both occasions. Five *Periplaneta* nymphs were used, four *Blatta* and four *Blaberus*. The *Blaberus* nymphs which had been fed with *Blatta* faeces were kept in oxygen at $3\frac{1}{2}$ atmospheres pressure for 21 hr.; all other nymphs were treated for 12 hr.

Results. The results were disappointing. All the *Nyctotherus* in all but one of the nymphs were killed by the treatment—hence, there was no apparent difference between the populations from different sources. However, *Nyctotherus* survived in good numbers in one of the *Blatta* which had been fed faeces from *Periplaneta*.

Conclusions. A treatment period of 12 hr. was enough to kill *Nyctotherus* in *Periplaneta*: the fact that the *Nyctotherus* survived in the *Blatta*, which was infected with *Nyctotherus* from *Periplaneta*, indicates that the increased resistance resides in the host and is not a peculiarity of the *Nyctotherus* population itself. This is supported by other evidence set out in the second and in the third papers of this series which describe observations on the cyst size of *Nyctotherus* and on excystation in *Nyctotherus*: there seems to be no genetic difference between the populations of *Nyctotherus* in the different species of cockroaches, although they may differ greatly in appearance. This is also supported by the results reported in the latter part of Cleveland's (1925*b*) paper. He found that *Trichomonas* in culture is killed in about half the time that it takes to kill it in the frog, and sug-

gested that the host forms some sort of barrier between the oxygen and the Protozoa. The nature of this barrier is obscure: it was most efficient in *Blaberus* and *Blattella*; the former is large and generally torpid, the latter is small and very active.

Cleveland also tested the effect of an increased pressure of oxygen on some free-living Protozoa and found that it is as toxic for some as for parasitic forms. He pointed out that Paul Bert reported numerous experiments as long ago as 1874, which showed that excess oxygen was toxic for many or perhaps all forms of life.

More recently, Stadie, Riggs & Haugaard (1944) reviewed the literature on the general biochemical effects of excess oxygen on metabolic pathways.

Gut parasites are likely to be handicapped by a lack of enzyme systems capable of dealing with anything but the very low oxygen tension of their natural environment. In this connexion, Laser (1944) showed that *Ascaris suis* has very little cytochrome. Pure oxygen is lethal because there is too little catalase to disperse the accumulation of hydrogen peroxide.

The different degrees of resistance to oxygen poisoning shown by *Monocercomonoides*, *Endamoeba*, *Lophomonas* and *Nyctotherus* was in strong contrast to the very similar degrees of resistance shown by each of the thirteen genera of Protozoa in *Cryptocercus*, found by Cleveland & Burke (1956). There is no obvious reason for the difference between the endoparasitic Protozoa of cockroaches.

III. THE EFFECT OF MOULTING ON THE INTESTINAL PROTOZOA

(*a*) *Introduction*

A nymph which is about to moult can be recognized by the fatness and opacity of the wing pads, which is caused by the development of the new pads, or complete wings, within the old. Its gut contents are as described below: evidently this condition arises some few days before the final emergence takes place. Once emergence is completed, the new cuticle tans to the colour of the old and the state of the gut contents returns to normal within 24 hr.

Dissection of cockroaches which were moulting showed that both the fore- and mid-gut were usually empty of food. The posterior part of the hind-gut was often distended with air and there was a small amount of nearly desiccated and almost leathery matter in the anterior part. This consisted of some food debris and fragments of the old peritrophic membrane. There might also be a small population of protozoan and nematode parasites. The protozoan which seemed to occur to the exclusion of nearly all others was *Nyctotherus ovalis*. Its shape and that of the macronucleus was often greatly distorted: its appearance will be described more fully later.

(*b*) *The period of self-starvation*

Fourteen nymphal *Periplaneta* were selected which had fat, opaque wing-pads. Eight *Blatta* were also taken, but the choice was more difficult here, because the wing-pads are always densely pigmented and do not seem to fatten to the extent

found in pre-moult *Periplaneta*. In the event, all fourteen *Periplaneta* moulted, but only three *Blatta*.

Each nymph was given a scrap of lettuce daily. Most of the *Periplaneta* moulted in 3 or 4 days without eating lettuce. However, two nymphs underwent a defined period of starvation and finally moulted. The period was 9 days in one and 10 in the other. Similarly, two of the *Blatta* underwent defined periods of 2 and 9 days' starvation respectively.

These few observations suggest that, before moulting, the large or final instar nymphal *Periplaneta* and *Blatta* undergo a period of voluntary starvation which may last for 9 or 10 days.

(*c*) *The position of* Nyctotherus *in the hind-gut*

Observations, which are not reported here, showed that in both *Periplaneta* and *Blatta*, the preferred habitat of the trophozoites of *Nyctotherus* in the hind-gut is well anterior to the anus. (The hind-gut was divided into five sections, no. 1 being most anterior: the largest numbers of *Nyctotherus* were found in sections no. 2 and nearly as many in no. 3.) Many dissections of *Blaberus* in the course of other experiments showed that *Nyctotherus* occupies approximately the same position in the hind-gut of this species also.

Two or three of each species of cockroach were dissected at or very soon after a moult. The hind-guts were divided into five sections as before, and the position of the *Nyctotherus* determined.

In all cockroaches, the largest numbers were found in sections no. 3 and 4. This suggests that *Nyctotherus* moves either passively or actively down the hind-gut towards the anus at the time of moulting. The position of the cysts in the hind-gut was determined also: their distribution seemed to be the same as in non-moulting cockroaches. If the movement of the trophozoites had been passive, the cysts ought to have moved too. Evidently, then, there is a tendency for the trophozoites to migrate towards the anus.

(*d*) *The appearance of* Nyctotherus

The shape of the trophozoites and of the macronucleus within each tended to be greatly distorted. In *Periplaneta* this seemed to be invariably so. The least change in the shape of the body was from a flattened pear-shape to a relatively much fatter and rounder form. Some of these moved sluggishly but many were immobile, although their cilia were still beating slowly. The shape of the macronucleus in these animals might be the normal rounded triangle; but in some, the macronucleus was stretched across the body. At the other end of the scale, many trophozoites had lost their smooth outline, being either lumpy or polygonal. The macronucleus was usually stretched transversely; and the cilia were beating slowly or had often stopped altogether.

Careful dissection of the hind-gut contents showed that the polygonal shapes were due to the trophozoites being tightly balled together, the whole giving the appearance of a raspberry or an embryonic morula. Each was pressed so closely by its neighbours that its shape suggested an irregular crystal.

This massing together of *Nyctotherus* did not occur in every cockroach: it did not occur when there were few trophozoites in the gut, and in any one case never involved all the trophozoites. A proportion stayed outside the group, adopting the simple spherical shape and might be still moving at the time of moulting.

This was also illustrated by two *Blaberus* which were dissected. One contained a large number of *Nyctotherus*: in one section of the hind-gut all were deformed and dead and some decayed; in another, many were grouped tightly together and were still alive; and in a third section, a few were mildly deformed and still swimming. In the second *Blaberus*, the hind-gut contents was a little moister and there were few *Nyctotherus*. All were moving sluggishly, were mildly deformed and a few of them had a macronucleus which was stretched transversely.

This condition also occurred in *Blatta*, but less frequently. The hind-gut contents in this cockroach tended to be moister than in *Periplaneta* or *Blaberus* at the time of moulting, and this seemed to be related to the less frequent occurrence of the grossly deformed *Nyctotherus*.

Whenever it occurred, the 'raspberry' was examined to see if the trophozoites were adhering closely to each other and therefore whether exchange of nuclear material was possible. In every case it was possible to break up the group very easily—in fact this tends to happen if the dissection of the hind-gut contents is not careful.

This 'raspberry' has been found in most but not all *Periplaneta* in a pre-moult condition and therefore must have been formed at least one and maybe, two or more days before the cockroach emerged. It was found in *Periplaneta*, *Blatta* and *Blaberus* which have moulted recently. It seemed to persist for up to 8 hr. and was followed, during the next 8 hr. or less, by the resumption of normal shape by the individual trophozoites. These times apply to cockroaches kept without food and water, and might be shorter in nature.

The macronucleus also resumed its normal shape at about the same time as the body. The way in which it was distorted at the time the host moulted is of importance because it revealed an unsuspected attribute of its form. The macronucleus was a folded sheet: the two halves lay neatly one on top of the other in the normal trophozoite. When the host moulted, the half-sheets flattened and swivelled apart, first into a stubby V, with the two arms pointing posteriorly; and then this might open out still further until the sheet was completely unfolded and lay stretched right across the body or canting diagonally. After the moult, the macronucleus resumed its normal shape by refolding.

The changes in shape of the macronucleus and of the body after the moult are illustrated in Figs. 1 and 2. These histograms were compiled from seventy-five specimens of *Nyctotherus* found in the hind-gut of ten *Periplaneta* killed within 1 hr. of moulting ('at moult'), and seventy-two specimens found in six *Periplaneta* 18–30 hr. after moulting ('post-moult'). In each case, the hind-gut was dissected out, the contents mixed with a drop of egg albumen or of horse serum and smeared out on a slide. The smear was fixed in osmic acid vapour and stained with Heidenhain's iron haematoxylin and eosin. Camera lucida drawings were made, and the length and breadth of the body and the greater and lesser dimensions of the

macronucleus were measured in each specimen. In all cases, the greater dimension of the macronucleus was measured as the sum of the lengths of the two arms of the V, and the lesser dimension as the maximum width measured at right angles to an arm of the V.

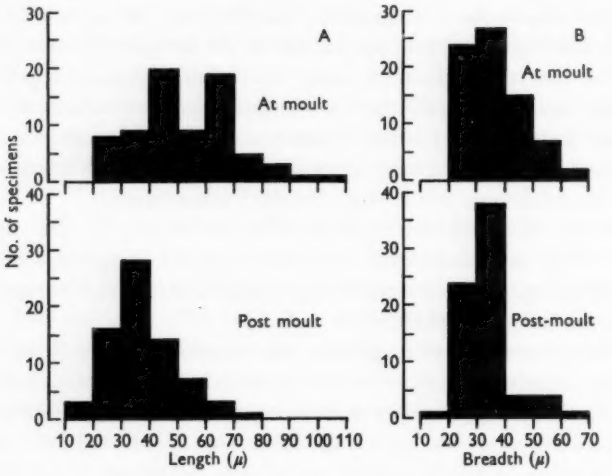

Fig. 1. The change in dimensions of the macronucleus of trophozoites of *Nyctotherus ovalis* in the hind-gut of *Periplaneta americana* when the host was moulting compared with when the host had completed moulting 18–30 hr. beforehand. (A) Changes in the greater dimension of the macronucleus, (B) changes in the lesser dimension.

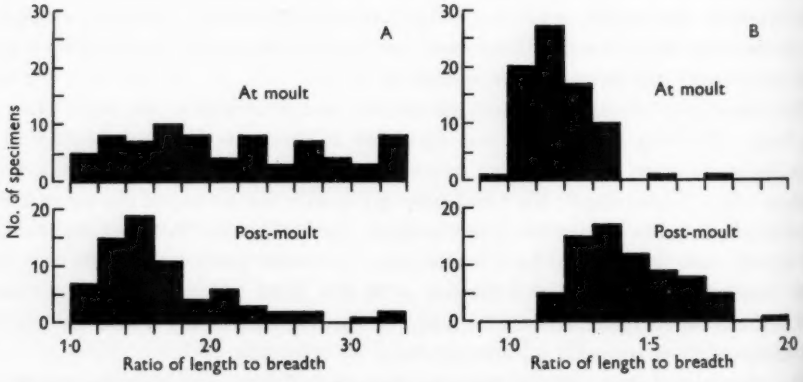

Fig. 2. The changes in the shape (ratio of greater to lesser dimension) of (A) the macronuculeus and (B) the body of trophozoites of *Nyctotherus ovalis* in the 'at moult' and the 'post-moult' *Periplaneta americana*.

Figs 1A, B and 2A relate to the change in shape of the macronucleus and show that at moult, the macronucleus was stretched along its greater dimension; there was also a stretching along its lesser dimension, but this was not so marked. In consequence, the unfolded sheet was relatively longer, as well as being larger (flatter).

Fig. 2 B shows that, at moult, the body is much rounder than normal.

Selected specimens are shown in Fig. 3. Specimens *a* to *h* are all from *Periplaneta* at moult: they are arranged in order of degree of unfolding of the macronucleus. Specimens *i* to *k* are from post-moult *Periplaneta* and show stages in the refolding of the macronucleus.

This change in shape of the body and macronucleus was also followed in individual living specimens. As I have already stated, the more distorted ones were immobile or nearly so, but later recovered. The difficulties of making accurate drawings when the ciliate starts to move again was partly overcome by mixing the

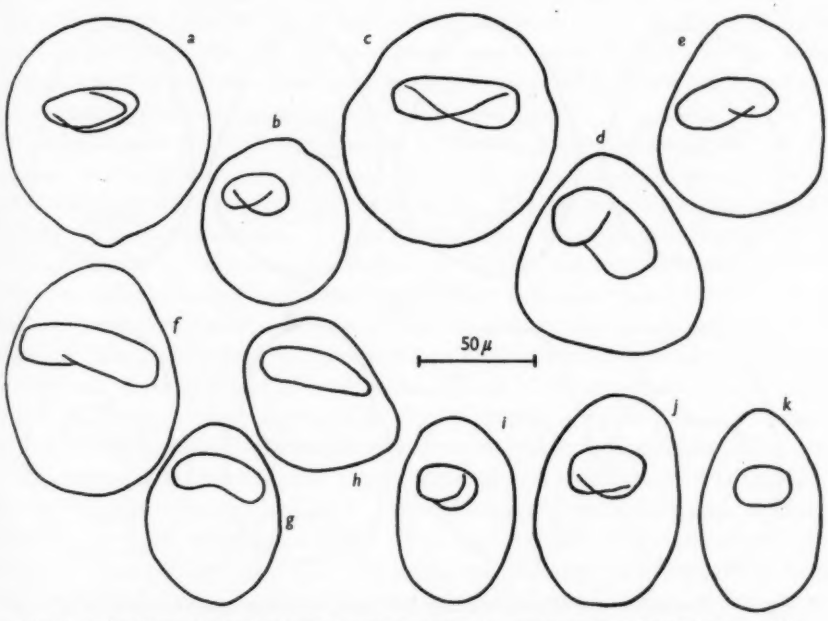

Fig. 3. The form of the body and macronucleus in specimens of *Nyctotherus ovalis* in *Periplaneta americana* 'at moult' (*a–h*) and 'post-moult' (*i–k*).

hind-gut contents with a concentrated extract of carrageen ('Irish Moss'. This sea-weed is boiled in a little water for a few minutes and yields a mass of thick mucilage). Further, vital staining of the macronucleus with a 0·01 % solution of malachite green makes it more distinct.

The high viscosity of the medium restrained the ciliate during the early stages of its recovery but was ineffective in stopping movement later on. In consequence, it was impossible to make a complete series of camera lucida drawings of recovery in any one specimen. Fig. 4 shows the sequence in one over a period of 70 min. It was from the hind-gut of a large nymphal *Periplaneta* which had just emerged from its old skin. The dissection, staining and mounting was carried through as quickly as possible, and the first drawings made at 'time 0'. There was no great distortion of the body, but it became slightly less round and more pear-shaped

as time went on. The macronucleus started to refold and the process was well advanced at the end of 70 min., when the ciliate finally swam off. The timing of the process was much shorter than those observed *in vivo*.

(e) *The fate of the other Protozoa*

Incidental to the observations made on *Nyctotherus*, some data were collected on the other Protozoa of the hind-gut. Twenty-five specimens of *Periplaneta* which were examined at moult appeared to be infected only with *Nyctotherus*. However, of twenty-one in a post-moult condition, sixteen were infected with

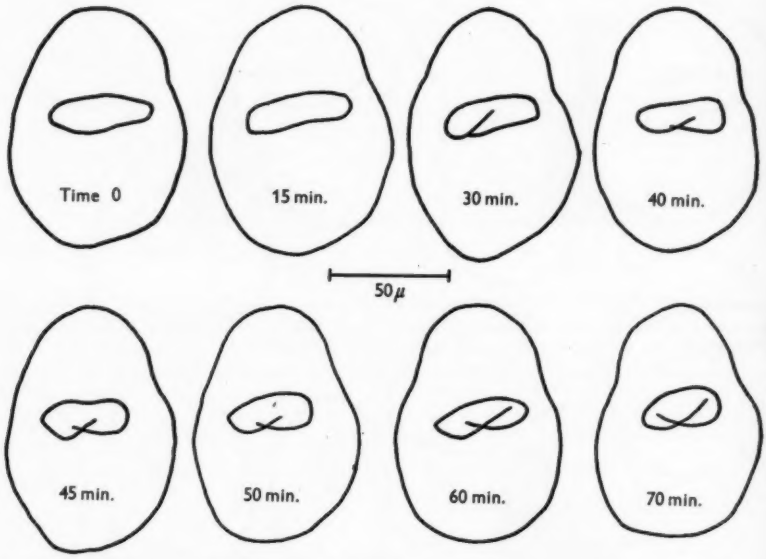

Fig. 4. The recovery of the shape of the body and the macronuculeus of *Nyctotherus ovalis* in the moulting cockroach observed *in vitro* for 70 min.

other Protozoa: *Lophomonas striata* in thirteen, *L. blattarum* in two, *Monocercomonoides* in ten and *Endamoeba* in one. The low incidence of the second and fourth species was probably partly due to the low percentage of infected animals in the stock as a whole. These figures strongly suggest that these Protozoa may survive the moult, along with *Nyctotherus*. Their absence from moulting cockroaches was therefore real in only some cases; but in others, they must have been overlooked. It is therefore probable that they were immobile and greatly changed in appearance.

The protozoa in *Blatta* did retain their normal appearance and motility in a few cases. Of eleven which were infected with *Nyctotherus*, two harboured a single recognizable specimen of *Endamoeba* each, but a third harboured twenty or thirty in a gut which was noticeably moister than usual. Cysts of *Endamoeba* were also present. But they were present in four others of the eleven from which

trophozoites appeared to be absent. Specimens examined in post-moult harboured *Lophomonas striata* and *Monocercomonoides*, in addition to *Nyctotherus* and *Endamoeba*.

(*f*) *Attempts to induce the changes in the appearance of* Nyctotherus

A possible explanation of the changes in the appearance of *Nyctotherus* and of the tendency towards defaunation is that they were merely secondary or indirect effects of moulting. The immediate cause might have been the change in the hind-gut caused by the self-imposed starvation. This would result in there being less material in the hind-gut and would reduce the water content of the material and of the whole body. The dryness of the medium might have led to the changes in shape of *Nyctotherus*, stopped its movements and tended to eliminate the other Protozoa; and the paucity of the medium might have led to the clumping of the ciliates when they were present in large numbers.

However, Armer (1944) in her account of the effect of extremes of diet on the Protozoa of *Periplaneta americana* mentioned no such effects of starvation on *Nyctotherus*, even if it was prolonged for 10 weeks. She did note, however, a general tendency towards defaunation: *Lophomonas blattarum* and *Endolimax blattae* were eliminated; *L. striata* and *Endamoeba blattae* were reduced in number; *Nyctotherus* was unchanged in number, but 'a small flagellate' increased in number. After only 2 weeks' starvation, these effects were, however, barely apparent.

Nevertheless, the idea that the changes were a result of starvation and dehydration was explored. Nymphal and adult *Periplaneta* and *Blatta* were denied food, water and prevented from eating their own faeces for periods of up to 9 days. A reduction in the number of *Nyctotherus* and of *Endamoeba*, *Lophomonas striata* and *Monocercomonoides* was noticeable after 6 days. The shape of *Nyctotherus* was normal.

Likewise, no success was had when hypertonic solutions of carrageen, sucrose, uncooked agar-agar, boiled or unboiled starch, or 'polycell' were injected into the hind-gut or into the fore-gut. (The last agent is a cellulose adhesive of the type recommended by Ellenby (1956) for arresting *Paramecium*.) Some of the *Nyctotherus* were still plasmolyzed when examined up to 18 hr. later, but all were mobile and the macronucleus was unaltered.

(*g*) *Discussion*

The failure to demonstrate a sexual stage in *Nycototherus ovalis* at the time of the moult is of some interest. Conjugation in *N. cordiformis* was described by Bojewa-Petruschewskaja (1933) and in greater detail by Wichterman (1937). Both noticed that it occurred at the time that the frog metamorphosed from tadpole to adult frog. Bieniarz (1950) and Čehović (1956) claimed that cysts are found in frog's faeces only in the breeding season, that these cysts are picked up by tadpoles in which they hatch, and that it is the newly excysted forms which conjugate. In effect, they asserted that the cyst is the first stage in the sexual cycle of *N. cordiformis*. Wichterman found, however, that cysts are produced by tadpoles as well as by adult frogs, and that the process of excystation is irrelevant to conjugation: if excystation occurs in a tadpole, several mitotic divisions can

occur before conjugation. This version of the life-cycle is supported by the work of Aragão (1912). He succeeded in obtaining encystation of *N. cordiformis* in culture. (Bieniarz was unable to induce encystation in culture, even when he added pregnancy urine to the medium.) The difference between these versions of the life-cycle suggests that either there is some variation in the occurrence of cysts of *N. cordiformis* in different stocks of frogs, or that one pair of authors was dealing with another species of *Nyctotherus*. The latter is certainly true of the form observed by Higgins (1929): it was like *N. cordiformis* but lacked the oral bristle: its size varied in the tadpole more than in the adult frog, and she suggested that the variations may be immature or larval stages.

All authors were agreed that metamorphosis is accompanied by conjugation of the parasite. This gives some grounds for expecting moulting in the cockroach to be accompanied by conjugation in *N. ovalis*. But even the changes in the macronucleus cannot be regarded as a rudiment or a vestige of the process: they bear no resemblance to the fragmentation and reorganization described by Wichterman (1937).

The absence of conjugation in *Nyctotherus* of the cockroach, but occurrence in that of the frog, is paralleled by the absence of sexual stages in the Protozoa of termites, but occurrence in similar forms in the wood-eating roach, *Cryptocercus punctulatus*. The latter have been described in great detail by Cleveland (1949, 1950*a–d*, 1951*a*, *b*, 1952, 1953, 1954*a–c*; 1955, 1956, 1957. Cleveland (1959) has also shown that the sexual cycle of the Protozoa of *Cryptocercus* is influenced by the moulting hormone, ecdysone, of the host. In addition to inducing the gametogenesis phase of the sexual cycle in the protozoa of *Cryptocercus* ecdysone may, in some instances, accelerate or retard sexual processes such as fertilization and zygotic meiosis (Cleveland, Burke & Karlson, 1960).

There is insufficient evidence to show if any of the changes in the gut Protozoa of cockroaches were caused directly by the moulting hormone. Certainly, the tendency towards defaunation could have been merely an effect of the self-starvation. The immobility and change in the appearance of *Nyctotherus* and the other Protozoa might have been an effect of the dehydration of the environment, but there was no experimental evidence to support this. The failure to get this evidence may have been due to the treatments being too harsh and rapid: they could not be otherwise, because the normal cockroach is able to supply the water requirements of a gut which is artificially slightly dehydrated. The unfolding of the macronucleus was probably merely a slight breakdown in the physical organization. Pai & Wang (1948) noted considerable variation in the shape of the macronucleus, but apparently never saw the folded structure. The observations of Kidder (1937) on *N. uichancoi* in the wood-feeding roaches *Panesthia javanica* and *P. spadica* show that in this species the macronucleus is always in a partly unfolded state. Viewed from the oral or from the aboral end (that is, 'end-on'), the macronucleus is in the shape of a boomerang, lying over the cytopharynx.

No change was ever seen in the micronucleus, which is of greater genetic importance. How, or why, moulting hormone in *Cryptocercus* or metamorphosis hormone in the frog stimulates the parasites to exchange their genes remains obscure.

IV. SUMMARY

Records of non-sporozoan Protozoa occurring in the hind-gut of cockroaches are summarized.

The Protozoa studied were the three flagellates *Lophomonas striata*, *L. blattarum*, *Monocercomonoides orthopterorum*; the rhizopod *Endamoeba blattae*, and the ciliate *Nyctotherus ovalis*, in the cockroaches *Periplaneta americana*, *P. australasiae*, *Blatta orientalis*, *Blattella germanica* and *Blaberus giganteus*.

Specimens of *Blatta* and *Periplaneta americana* were maintained at 37° and 25° C. under extreme conditions of humidity. Maximum humidity enabled the host, and the *Nyctotherus* which it harboured to survive for a longer time. The results of the experiments indicated that the well-being of the protozoan parasites is closely linked to that of the host.

Periplaneta americana, *Blatta*, *Blatella* and *Blaberus* were subjected to pure oxygen at 3½ atmospheres pressure and the effect on the Protozoa observed. The flagellates were killed first, then *Endamoeba* and lastly *Nyctotherus*. They survived in *Blatta* for a longer time than in *Periplaneta*; and *Nyctotherus* in *Blaberus* and *Blattella* sometimes survived the hosts. The hosts appeared to act as a barrier against poisoning by oxygen, and the strength of the barrier varied in the different species of cockroaches.

Nymphal *Periplaneta* or *Blatta* which were about to moult underwent a period of self-starvation which lasted for up to 10 days. The period was terminated by the moult: at this time, the little food that was in the gut was very dry. All species of Protozoa could survive these conditions, but *Nyctotherus* seemed to be the most hardy. The main mass of ciliates seemed to be nearer to the anus than in the non-moulting cockroach. Many of the ciliates were often clumped together into a 'raspberry'. The bodies of the individual ciliates were more spherical than usual, but those in the 'raspberry' were moulded into polygonal shapes. The macronucleus underwent a change in form which revealed that its basic structure is that of a folded sheet.

The changes were reversed within 24 hr. They could not be induced by experimentally imposed conditions of starvation or dehydration of the hind-gut. The changes in *Nyctotherus ovalis* are contrasted with the sexual stages occurring in *N. cordiformis* and in the Protozoa of *Cryptocercus*.

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The protozoa occurring in the hind-gut of cockroaches

II. Morphology of *Nyctotherus ovalis*

By H. M. D. HOYTE

*Molteno Institute, University of Cambridge**

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I. THE SIZE AND SHAPE OF THE TROPHOZOITE

(*a*) *Introduction*

The name *Nyctotherus velox* was given by Leidy (1849) to a ciliate which he found in the hind-gut of the millipede *Julus marginatus*. In the following year, he added a second species to the new genus: *N. ovalis*, which he found in a similar site in the cockroach *Blatta orientalis*. Since that time, more than fifty-five forms have been described from various hosts, and most were designated as new species. Bhatia & Gulati (1927), Zeliff (1933), Rosenberg (1937) and Wichterman (1937) listed the species in increasing number. Some additional ones were mentioned by Reichenow (1953). Additional records are those by Carini (1938), de Puytorac (1954), Schouten (1940*a*, *b*), Semans (1941), Uttangi (1951) and Yamasaki (1939).

One of the criteria used in differentiating between species is the host of origin. In this connexion, it is interesting to note that some hosts are reputed to harbour more than one species. One is an unnamed wood-cockroach: Pinto (1926) found

* Present address: University of Queensland Veterinary School, Brisbane, Australia.

that it harboured *Nyctotherus buissoni* and *N. viannai*. Two other hosts of this sort are *Bufo melanostinctus* (*N. cordiformis* Ehrenberg and *N. papillatus* Dobell) and *Rana tigrina* (*N. macropharygeus* Bezzenberger, *N. papillatus* Dobell and *N. piscicola* Dobell). The other criteria which are used include the size of the parasite, its shape, the form of the cytopharynx, the shape of the macronucleus and the presence or absence of a caryophore. Variations in *N. ovalis* in some of these respects are described in the following pages.

(*b*) *Description*

The trophozoites of *Nyctotherus ovalis* varied in shape, when seen in the customary side view, between two extremes: in *Blatta orientalis* they were fat ovoids; in *Blattella germanica* they were like pears. Kudo (1936), who studied *Nyctotherus ovalis* in *Blatta*, pointed out that Stein (1867) was wrong in maintaining that the trophozoite is dorso-ventrally flattened. The free-swimming trophozoite is pyriform when seen in oral or aboral view, with sides which are slightly concave near the middle of the body. The flattening of the body which Stein observed, the appearance of his figures and the measurements which he gave of his specimens suggest that his specimens were compressed. Probably he omitted to provide support for the coverslip.

The shape seems to be more or less constant from individual to individual in any one population. Pai & Wang (1948) found that the correlation coefficient of length and breadth of trophozoites from *Periplaneta americana* was 0·99. The length varied from 55 to 105 μ and the breadth from 40 to 65 μ. The forms which Kudo observed in *Blatta* were much larger: 90–185 by 62–95 μ, with some giant forms which measured 320–360 μ long and 200–240 μ wide. Lucas (1927) mentioned that free-forms range up to 350 μ in length. Kudo noticed that the proportion of these giant forms increased when the hosts were fed on Difco egg-meat medium or yeast extract, or on yeast cakes, and inferred that the richer diet was responsible. This aspect was dealt with by Armer (1944) in some detail: she fed *Periplaneta americana* on a high-carbohydrate diet (cornmeal), a high-fat diet (vegetable fat) or a high-protein diet ('Protein Milk Powder'), and a fourth group was starved. The average size of the trophozoites increased only in those on the high-carbohydrate diet, from $73{\cdot}5 \pm 2{\cdot}0$ by $52{\cdot}2 \pm 1{\cdot}8$ μ to $91{\cdot}4 \pm 3{\cdot}7$ by $67{\cdot}5 \pm 2{\cdot}9$ μ. She did not give the range of size nor remark on the presence or absence of giant forms.

Like Kudo, I have seen these forms in cockroaches fed on a balanced diet. I have also seen them in *P. americana*, *Blattella germanica* and *Blaberus giganteus*; and also in cockroaches fed on diets deficient in carbohydrates. This suggests that these ciliates turned into giant forms not simply through over-feeding, but rather that there was some other factor responsible for a failure to initiate division at the normal time. Growth only ceased when a steady state was achieved, imposed, perhaps, by some physical limit to metabolism. However, Lom (1956) demonstrated that they had not lost the ability to divide, and that they can give rise to normal-sized individuals.

The increase in average size observed by Armer and the increased proportion of giant forms observed by Kudo are probably distinct phenomena. The richer

diet seems to result in an increased size at all stages of growth, and secondly, predisposes towards the failure to divide.

A type of cell-inclusion which has been observed in *Nyctotherus ovalis* by Lucas (1928), Kudo (1936), Armer (1944) and Lom (1955), in *N. silvestrianus* by Kirby (1932) and *N. uichancoi* by Kidder (1937) are the 'elliptical or spindle-shaped bodies', the 'glycogenous bodies', the 'paraglycogen granules', the 'zrny

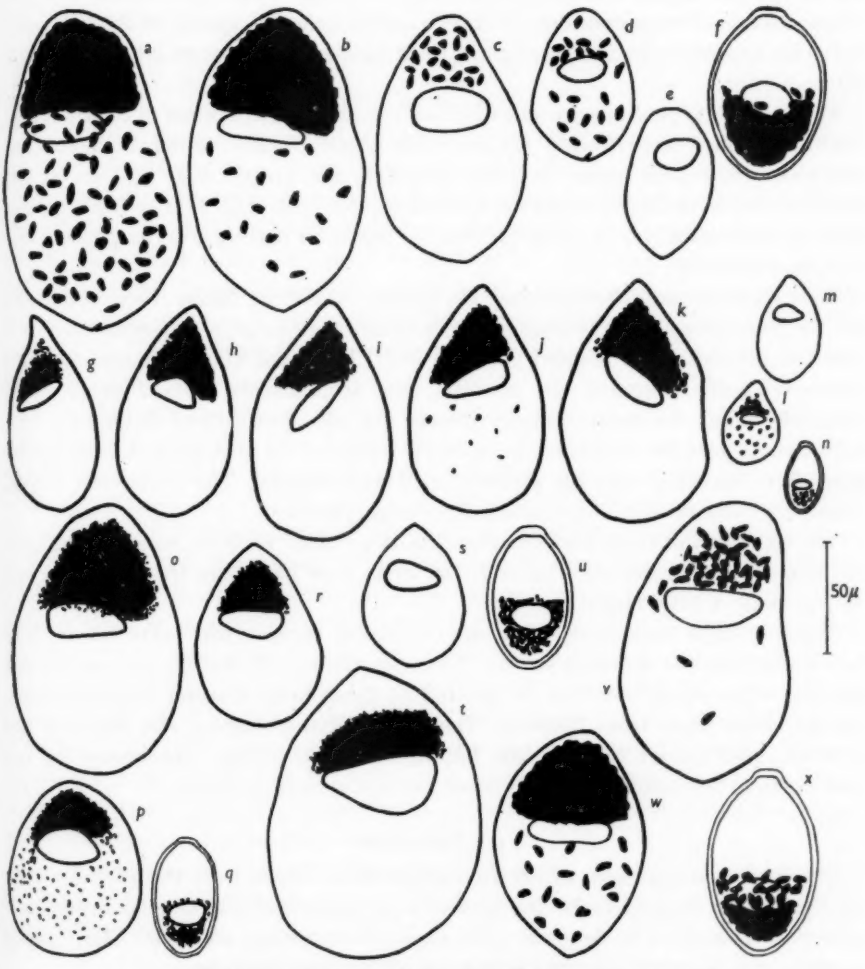

Fig. 1. The form of trophozoites and cysts of *Nyctotherus ovalis* in four species of cockroach. Specimens were freshly stained with Lugol's iodine solution which stained the paraglycogen granules dark brown. Specimens (*a*) to (*f*) from *Blatta orientalis*, (*g*) to (*n*) from *Blattella germanica*, (*o*) to (*q*) from *Blaberus giganteus*, (*r*) to (*u*) from *Periplaneta americana* of one stock and (*v*) to (*x*) from *P. americana* of a second stock. Note, for uniformity, the granules within the cysts (*f*, *n*, *q*, *u* and *x*) are coloured black, although the iodine did not penetrate the cyst walls.

paraglykogenu', the 'glycogen-like platelets', and 'ovoid refractile granules' respectively. Armer noted that when cockroaches are fed on a high-carbohydrate diet, not only is there an increase in the average size of the trophozoites, but there is also an increase in the numbers of paraglycogen granules within each. Likewise, Lom (1956) found that *N. ovalis* cultured in starch-deficient media were smaller and contained fewer paraglycogen granules than those maintained in richer media. These findings suggest a cause and effect relationship. But I have found that the concentration of these granules also varies according to the species of the host, and that this appears to be independent of their diet, since they were all fed on much the same diet.

Fig. 1 shows typical specimens of *N. ovalis* from the four species of cockroaches. All trophozoites were freshly stained with Lugol's iodine, which coloured the granules brown (this agent does not penetrate the cysts). Fig. 1(*a*) to (*e*) are trophozoites from *Blatta orientalis*; (*f*) is a typical cyst. Fig. 1(*g*) to (*n*) are from *Blattella germanica*, (*o*) to (*q*) from *Blaberus giganteus* and (*r*) to (*x*) are from *Periplaneta americana*.

Four main features characterized the trophozoites from *Blatta*: their large size, the frequent presence of storage granules which were large, the rounded anterior end and the somewhat pointed posterior end. The cysts were large and fat, and contained large granules. In contrast, the trophozoites from *Blattella* were relatively small, contained no large storage granules, but instead frequently contained small granules with the same staining reaction; the anterior end was pointed and often beak-like, and the posterior end was rounded. The cysts were small, relatively more slender and contained no large granules.

The trophozoites from *Blaberus* were like those from *Blattella*, except that they did not have a pointed anterior end. The cysts were like those from *Blattella* but were usually a little larger.

The specimens from *Periplaneta* are of interest because they were taken from two cockroaches of different stocks. Those specimens labelled (*r*), (*s*) and (*t*) had anterior ends which were not so pointed as those from *Blattella* but were more pointed than those from *Blaberus*. Their other characteristics, and those of the cyst (*u*), also placed them in the *Blattella–Blaberus* group. Specimens (*v*), (*w*) and (*x*) from the other cockroach, were like the ones from *Blatta*.

(*c*) *Discussion*

The main characteristic which distinguished the *Blatta* from the *Blattella* type of *Nyctotherus* seemed to be the presence or absence of the large paraglycogen granules. It seemed to determine the sizes of trophozoite and cyst. The pointed anterior end occurred only in the absence of the large granules.

Trophozoites which contained these large granules were harboured by both my stocks of *Blatta*, and I have seen them in samples of other stocks of this species. They also occurred in one, but not the other of my stocks of *Periplaneta*. I have never seen this type of *N. ovalis* in *Blattella* or in *Blaberus*. This distribution suggests that *Blatta* supplied some factor which induced the parasite to store paraglycogen in the form of large granules, rather than in the form of the small granules which

were typical of those in *Blattella* and *Blaberus*. But it is difficult to believe that one population of *Periplaneta* supplied this factor and another did not. The effect, if indeed there was a host-effect, must have been indirect. An hypothesis to account for the facts is that some member of the flora in the gut of the cockroach supplied the factor. This member was typically present in *Blatta*, and could be present in a population of *Periplaneta*, but was typically absent from *Blattella* and *Blaberus*. Some further evidence which supports this hypothesis is reported in the Part on the sizes of the cysts.

II. THE SIZES OF THE CYSTS

(*a*) *Introduction*

The cysts of the various species of the genus *Nyctotherus* are egg-shaped, but those of *N. ovalis* are unusual in having, at the blunt end, a knob. (During the final stages of excystation, this knob lifts and the ciliate emerges through this opening.) Three papers give the data on the measurements of the cysts of *N. ovalis*: Lucas (1927) stated that 'cysts range only from 55 to 94 μ in length and from 25 to 70 μ in greatest width'. Pai & Wang (1948) found cysts which ranged from 55 to 105 μ in length and from 40 to 65 μ in width. They were preceded by Stein (1867) who stated that 'Der grössten Cysten waren 1/18‴ lang und 1/30‴ breit, die gewöhnlichsten 1/30–1/24‴ lang und 1/40–1/30‴ breit, die kleinsten 1/35‴ lang und 1/54‴ breit'. Although his statement is more detailed, there is some doubt as to the meaning of these figures, since it depends on the definition of his units of measurement. The mark ‴ is the abbreviation for the German 'Linie', but German scientists used the 'Pariser Linie' (1‴ = 2·2558 mm.) rather than their native one (which varied in size in different parts of Germany). In 1845, some twenty years prior to Stein's publication, the Pariser Linie was generally abandoned in favour of millimetres.* Nevertheless, since Stein nowhere stated what he meant by ‴, it must be assumed that he was still using the old unit of measurement. (Wichterman (1937) was faced with this same problem of interpretation when he translated Stein's measurements of *N. cordiformis*. He evidently took 1‴ to equal 2·117 mm., i.e. he assumed that Stein was using the English 'line'. Kudo (1936) assumed 1‴ equals 2·180 mm.: one of the German 'Linie'.) Assuming that Stein did use the 'Pariser Linie', the largest cysts were 125 × 75 μ, the average were 75–94 × 56–75 μ and the smallest were 64 × 42 μ. The cysts that he saw were on the whole, therefore, larger than those seen by Lucas or by Pai & Wang.

Lucas obtained her material from *Blatta orientalis*, and Stein from the same species and from *Blattella germanica*. Pai & Wang obtained their material from *Periplaneta americana*.

(*b*) *Day-to-day fluctuations in cyst size*

Material. Cysts of *Nyctotherus* contained in the faeces of an adult female *Periplaneta americana*.

Methods. The cockroach was isolated in a 3½ × 3 in. glass pot, the floor of which was covered with wet filter-paper. It was kept supplied with apple, horse-heart

* Based on a personal communication from Professor Dr H. Meyer, Physikalisches Staatsinstitut, Hamburg.

meat and rat-cake. The faeces were collected daily, macerated in a few drops of 0·5% sodium chloride solution, and the length and breadth of about thirty of the cysts measured. The maceration was made easier because the faeces, having been dropped on to wet filter-paper, were still soft.

Results. The faeces were collected and a sample measured daily for 1 month (28 days). On the tenth and twenty-sixth days the cockroach did not defaecate. (A daily production of one or more pieces of faeces was normal for all cockroaches of this and the other species kept under these conditions.)

The shortest cyst which was found measured 41 μ; the longest measured 69 μ. The range of lengths covered by each daily sample of cysts was never less than 12 μ but was usually about 17 μ. Similarly, the narrowest cyst measured 24 μ, the broadest 46 μ; but the usual range was about 11 μ.

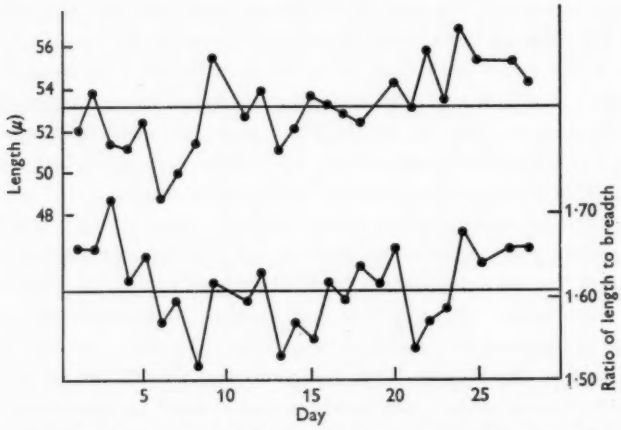

Fig. 2. Day-to-day fluctuations in the average size (upper chart) and shape (lower chart) of the cysts of *Nyctotherus ovalis* in the faeces of a specimen of *Periplaneta americana* kept under constant conditions of food, temperature and humidity. The horizontal line through each chart represents the average size and shape for all the daily samples.

The average length and breadth was calculated for each sample of thirty cysts. The upper chart in Fig. 2 represents the successive fluctuations in the average length; and the lower chart shows the fluctuations in the average ratio of length to breadth. The horizontal line through each chart represents the overall average around which the daily averages fluctuated. They were 53·1 μ and 1·61 μ, with standard deviations of 1·97 and 0·049 respectively. 73% of the figures for the daily average lengths lay within 1 Standard Deviation of the overall average, and 96% lay within 2 Standard Deviations. The corresponding proportions of the daily average ratios were 77 and 96%.

There seems to be no close relationship between the fluctuations in the average length and the average ratio of length to breadth. The tendency for both to rise from the thirteenth day onwards is suggestive, but this was shown not to be a substantial trend. Samples of cysts were taken two weeks and six months after the

end of this series, and had average lengths of 56·7 and 54·8 μ, and average ratios of 1·61 and 1·65 respectively.

These charts show that, despite the large range of size of the cysts within each sample, the average size and shape remained relatively constant from day to day.

(*c*) *Cyst size in relation to the species of host*

Material. Cysts of *Nyctotherus* contained in the faeces of several adult specimens of *Periplaneta americana*, *Blatta orientalis*, *Blaberus giganteus* and *Blattella germanica*.

Methods. The same procedure was followed as in section (*b*), except that only a few daily collections of faeces from each cockroach were used.

Results. The shortest cyst found throughout this series was 40 μ in length; the longest measured 118 μ. However, as was expected on the basis of the observation described in section (*b*), the cysts appearing in the faeces of any one cockroach varied from day to day around a more or less constant average size and shape.

Table 1. *Size and shape of cysts of* Nyctotherus ovalis *from four species of cockroach*

	Length (μ)		Breadth (μ)		Ratio of length to breadth	
	Range	Average	Range	Average	Range	Average
B. germanica	40– 56	46·8	22–28	24·8	1·68–2·33	1·90
B. giganteus	41– 64	52·7	25–37	31·5	1·47–2·00	1·66
P. americana (1)	41– 81	54·8	26–45	32·6	1·28–2·04	1·68
(2)	60– 97	78·0	42–68	51·9	1·31–1·96	1·51
(3)	55– 78	66·9	43–55	49·4	1·26–1·50	1·35
B. orientalis (1)	74–110	91·4	41–70	58·6	1·39–1·88	1·57
(2)	76–118	91·0	52–74	61·4	1·04–1·70	1·49
(3)	60– 91	73·0	43–67	53·8	1·12–1·51	1·36

Table 1 lists the ranges and averages of the lengths, breadths and ratios of length to breadth seen in samples of not less than 30 cysts contained in one day's faeces from a selection of the cockroaches. Data from only one *Blattella* and one *Blaberus* are given, because there seemed to be little variation in the cysts from cockroach to cockroach of either species. Data are given for three *Periplaneta*: no. 1 was from one stock, and nos. 2 and 3 were from a second. Likewise, *Blatta* nos. 1 and 3 were from one stock, and no. 2 was from a second.

The cysts from *Blattella* were the smallest, and were narrow ovoids. Those from *Blatta* were the largest and most nearly spherical. The most extreme examples seen from each measured 56×24 μ (a ratio of 2·33 to 1) and 77×74 μ (a ratio of 1·04 to 1) respectively. The cysts from the specimens of *Blaberus* were of the *Blattella* type, but were not so extremely small nor so narrowly ovoid. Those from *Periplaneta* covered nearly the whole ranges of size and shape; and these cockroaches were drawn from two separate stocks. The specimens of *Blatta* were also drawn from two separate stocks, but the cysts in their faeces were found to cover only the middle and upper parts of the ranges in size and shape.

(d) Cyst size in relation to changes in the environment

The average size and shape of the *Nyctotherus* cysts in the faeces of a cockroach remains more or less constant. This was shown, in the observations recorded in secton (*b*), to be true of a cockroach kept under constant conditions. Radical changes in the environment of the ciliate might be expected to alter the characteristic average size and shape of the cysts, either by modification of the phenotype, or perhaps by selection. Such changes can be classified conveniently into those which are variations in the immediate environment of the ciliate, and those which are variations in the environment of the cockroach and which may indirectly affect the Protozoa of the gut. Two variations of the first type are: the changes occurring when the host moults, and variations found in the gut of one cockroach as compared to that of another. A variation of the second type is that due to the temperature of the host's environment.

A. *Moulting*

Only four sets of data were obtained because adult, rather than nymphal, cockroaches were the normal source of *Nyctotherus* cysts, since their faeces typically contain more cysts and adults typically defaecate more abundantly than do nymphs. Two of the sets refer to a pair of *Blatta*; two to a pair of *Periplaneta*.

The information derived from one specimen of *Blatta* is presented in Fig. 3. This set covered a period of 28 days, but was imperfect. No faeces were collected during a holiday period extending from the sixth to the first day before the moult took place, and this omission is represented in the figure by a broken line extending from days -6 to -1. However, the data derived from the other specimen of *Blatta* and from the two *Periplaneta* suggest that this loss of information on the size and shape of the cysts was not a real one, because, during the same period, cysts were absent from the faeces passed by the second *Blatta*, and the two *Periplaneta* did not defaecate. A second imperfection was that the daily samples of cysts which were taken before the moult took place, were too small for it to be possible to derive a useful average size and shape (faeces were scanty and contained comparatively few cysts). Therefore the daily samples are amalgamated in pairs, thus bringing the number of cysts in each double sample up to at least twenty.

The moult ('*m*' in the figure) was preceded by a period of at least 1 day and was succeeded by a period of 3 days when no faeces were passed. From $+4$ onwards, one or more pieces of faeces were passed daily. The average size and shape of the cysts contained in these faeces fluctuated from day to day, but there was no apparent trend, nor did these fluctuations exceed, by a significant amount, those found before the moult.

The second specimen of *Blatta* passed faeces from days -12 to -6 inclusive, and the average length and ratio of length to breadth of the cysts on 3 of these days was 70·1, 71·8, 72·4 μ and 1·38, 1·37, 1·36 respectively. (The faeces passed on the other days were used in routine experiments, because it was not then realized that the nymph was about to moult.) On day -5, no faeces were passed,

but on days −4, −3, −2 and −1 the cockroach did defaecate. The faeces were of a dry and leathery consistency, instead of being of the usual particulate semi-liquid nature. On 2 of these days, the faeces contained a few nematode eggs, but on no occasion were any cysts of *Nyctotherus* found. This indicates that there was an actual cessation of cyst production for a few days prior to the host's moulting. This also shows that the cysts present in the faeces following the moult of the first specimen of *Blatta* and of the two *Periplaneta* were not merely ones which had stagnated in the hind-gut since sometime before the moult, but were probably all true products of the post-moult population of *Nyctotherus*.

After the moult, no faeces were passed on days +1, +3 and +4. However, a small quantity was passed on day +2, and the cysts in this, together with those in

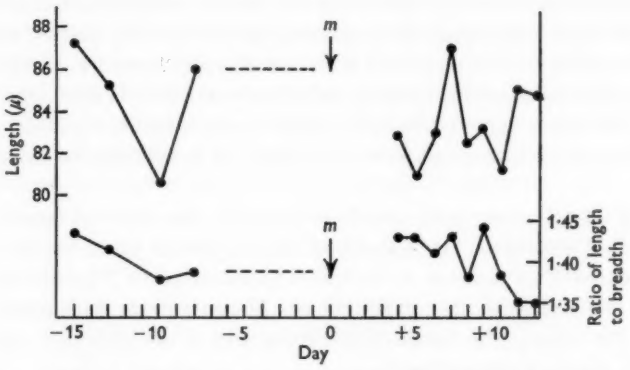

Fig. 3. The daily average size (upper chart) and shape (lower chart) of cysts of *Nyctotherus ovalis* in the faeces of a *Blatta orientalis* before and after the host moulted (*m*). For further explanation, see text.

the faeces of day +5, had an average length of 69·1 μ and an average ratio of length to breadth of 1·40. These averages differ from those derived from the pre-moult cysts by an insignificant amount.

The first specimen of *Periplaneta* did not defaecate for 4 days prior to moulting, nor on the 2 subsequent days. The average figures for a sample of cysts in the faeces on day +3 were 54·8 μ and 1·68. These are very little different from the averages (53·3 μ and 1·62) obtained from some cysts in faeces passed on a day 5 weeks before the moult took place.

The second specimen of *Periplaneta* did not defaecate for 5 days before, nor on the day immediately after moulting. However, faeces were passed on day +2, which contained a large number of cysts. The average figures of a sample of these were 78·0 μ and 1·51. The average figures, derived from three samples of suitable size, of cysts contained in the faeces passed during the four weeks prior to the moult were 80·8, 80·5, 76·9 μ and 1·46, 1·52, 1·47 respectively. No faeces were passed on days −7, −8, −9, −12 and −21; and even on the days when the nymphs did defaecate, the faeces contained only a few cysts.

This phenomenon, on a lesser scale, was also noticed in the first specimen of

Blatta and the first of *Periplaneta*. The faeces passed after the moult contained more cysts than did those passed immediately before the moult. Also, these cockroaches seemed to defaecate more frequently after moulting. The latter fact shows that the cockroaches had increased their daily intake of food; and the relatively greater number of cysts in the faeces was an indication of a larger population of Protozoa in their hind-guts, rather than a mere temporary increase in the proportion which underwent encystment. The observations also show that the average size and shape of the cysts remains unaffected by the host's moult.

B. *Change of host*

The variations found in the gut of one cockroach as compared to that of another can be attributed to two sets of factors. First, those variations attributable directly to variations in the physiology of the hosts as determined by species, sex, age, diet, temperature of the environment and other factors; and secondly, those attributable to the flora. The flora itself may be modified by variations in the physiology of the host, but will be determined primarily by the types of micro-organisms with which the hosts have come in contact and their ability to flourish in the intestines of the hosts.

In each of the following tests, uninfected cockroaches were fed faeces from a host infected with *Nyctotherus*. It is assumed that sufficient cysts hatched in the uninfected cockroach to produce a 'daughter population' of *Nyctotherus* which was genetically identical with that in the donor. Measurements were made of suitable samples of the cysts in the faeces of the donor and of the recipient, but in the first test, only in those of the recipients.

Test 1. A heavily infected adult female *Periplaneta americana* was confined in a glass pot with approximately forty newly hatched uninfected nymphs of the same species. They were fed apple, horse-heart meat and rat-cake; and kept at 25° C, as were all cockroaches in the succeeding tests. The nymphs were augmented by some which hatched from egg-cases laid by the female. Two years and four months after the start of the test, when the survivors of the original nymphs had long since reached maturity and had themselves produced offspring, an adult male, an adult female and a large nymphal female were removed from this crowded pot and placed in individual pots with a supply of food. Their faeces were collected on three occasions and suitable samples of the cysts contained therein were measured. Calculations were made of the average length and ratio of length to breadth of the cysts in each of the three samples from each cockroach. The overall averages around which these averages fluctuated, and the standard deviations, are noted in the following table:

	Length (μ)	Ratio of length to breadth
Adult male	$53{\cdot}9 \pm 0{\cdot}7$	$1{\cdot}56 \pm 0{\cdot}03$
Adult female	$51{\cdot}4 \pm 1{\cdot}8$	$1{\cdot}60 \pm 0{\cdot}04$
Nymphal female	$48{\cdot}8 \pm 0{\cdot}9$	$1{\cdot}47 \pm 0{\cdot}04$

By means of the *t* Test, these overall averages were tested against each other for statistical significance. Those cysts from the adult male and from the adult female did not differ from each other significantly in size or shape. However, those from the nymph, although not differing significantly from those of the adult female in size nor from those of the adult male in shape, did differ significantly in size from those of the adult male (level of significance between 1·0 and 0·1 %), and probably differed significantly in shape from those of the adult female (level of significance between 5 and 1 %). This suggests that there is an important variation in the gut of the host related to its age. This is put to the proof in the next test.

Test 2. Very young *Blaberus* were chosen as recipients because, in contrast to those of the smaller species, they could eat a reasonably large amount of faeces. Faeces were collected from an infected adult *Blaberus* and fed to an uninfected second-instar nymph of the same species. The nymph was kept in isolation and fed the same type of food as the adult received. One week after being fed faeces, its pot was cleared and, during the succeeding 5 weeks, three collections of its faeces were made. Samples of the cysts in each of these were measured and an overall average size and shape derived. These were compared with those obtained from three samples of the cysts in the faeces of the donor cockroach:

	Av. length (μ)	Av. ratio of length to breadth
Donor	52·5 ± 2·0	1·68 ± 0·04
Recipient	48·4 ± 2·9	1·57 ± 0·08
Difference	−4·1	−0·11
Level of significance	More than 5 %	More than 5 %

Thus, the age of the host appears to have no consistent effect on the size and shape of the cysts.

Test 3. The effect of variations determined by the species of the host was tested. An uninfected adult male *Periplaneta australasiae* was fed fresh faeces from an infected adult *Blaberus* and kept thereafter in isolation with a supply of food which was different from that received by the donor. (The *Periplaneta* received apple, lettuce, horse-heart meat and rat-cake; the *Blaberus* received lettuce only.) Faeces from the *Periplaneta* were collected during the subsequent 6 weeks, but no cysts were found after the first 4 days. It was therefore fed a second lot of infected faeces, but from another specimen of adult *Blaberus*. This time, an infection was established and measurements were made of three samples of cysts in faeces collected three, six and eight weeks after the second meal. The overall average size and shape of the cysts in the faeces from the *Periplaneta* are compared with those derived from three similar samples from the two *Blaberus*:

	Av. length (μ)	Av. ratio of length to breadth
1st donor	52·2 ± 2·2	1·61 ± 0·01
2nd donor	54·9 ± 1·8	1·68 ± 0·06
Recipient	45·5 ± 1·7	1·54 ± 0·04
Difference	−6·7 and −9·4	−0·07 and −0·14
Level of significance	Less than 1 % in both	More than 5 % in both

Six months after the first meal, another collection of faeces from the *Periplaneta* was made and a sample of the cysts measured. The average length and ratio were 46·3 and 1·53 μ respectively; indicating that the cysts maintained a stable average size and shape in a new host, after an initial significant decline in size: the new size of the cysts could be typical of the species of the new host, or it could be attributed to the difference in the diets received by the donors and the recipient. The latter hypothesis seems less likely because the *Periplaneta* received a richer diet than the *Blaberus* and therefore the Protozoa might be expected to be larger. In the next test, the recipient hosts were given a poorer diet than the donors.

Table 2*a*. *Change of size in* Nyctotherus *cysts in new hosts*

	Average lengths (μ)		
	Periplaneta I	*Periplaneta* II	*Blatta*
Donor	65·2 ± 1·4	73·1 ± 1·1	69·3 ± 1·6
Recipient *Blatta*	55·7 ± 1·0	42·2 ± 0·7	63·8 ± 2·1
Difference	− 9·5	− 30·9	− 5·5
Level of significance	Less than 0·1 %	Less than 0·1 %	Less than 5 %
Recipient *Periplaneta*	54·8 ± 1·0	—	61·1 ± 0·7
Difference	− 10·4	—	− 8·2
Level of significance	Less than 0·1 %	—	Less than 1 %
Difference between recipients	0·9	—	2·7
Level of significance	More than 5 %	—	More than 5 %

Table 2*b*. *Average ratios of length to breadth*

	Periplaneta I	*Periplaneta* II	*Blatta*
Donor	1·36 ± 0·04	1·43 ± 0·01	1·43 ± 0·02
Recipient *Blatta*	1·32 ± 0·02	1·78 ± 0·01	1·35 ± 0·01
Difference	− 0·04	+ 0·35	− 0·08
Level of significance	More than 5 %	Less than 0·1 %	Less than 5 %
Recipient *Periplaneta*	1·36 ± 0·04	—	1·49 ± 0·01
Difference	0	—	+ 0·06
Level of significance	—	—	Less than 5 %
Difference between recipients	0·04	—	0·14
Level of significance	More than 5 %	—	Less than 0·1 %

Test 4. Two adult *Periplaneta americana* and one adult *Blatta orientalis* were used as donors. Fresh faeces from each were collected and divided between an uninfected nymphal *Periplaneta* and an uninfected nymphal *Blatta*. This was repeated 4 days later. An infection with *Nyctotherus* was established in all but one of the six nymphs. (This one nymph had been given only a single meal of infected faeces, because it had eaten a large share on this occasion and there was insufficient on the second occasion for both nymphs.) As in previous tests, the nymphs were thereafter kept in isolation. They were fed only apple, whereas the donors received apple, lettuce, horse-heart meat and rat-cake. Two months after being fed faeces, a sample of faeces from each nymph was obtained by squeezing the abdomen. Two

more samples were obtained during the following week and a suitable sample of the cysts in each was measured. Similarly, three samples were taken from each of the donors and the overall average size and shape calculated for each cockroach. These averages are presented in Table 2*a* and *b*. (Those relating to *Periplaneta* I are based on six samples from the donor and five from each of the recipients, because those based on only three samples from each did suggest a possible significant difference in the sizes of the cysts from the two recipients.)

The most important of the results of this test was the size and shape adopted by the cysts from the *Blatta* recipient of faeces from the donor *Periplaneta* II. It was fed cysts which were large and rotund, but in turn produced cysts which were very small and comparatively very narrow: there was a change from the *Blatta*-type of cysts to the *Blattella*-type (see §(*c*) of this Part). This shows a continuity between these extreme types, and also shows that *Blatta*, like *Periplaneta*, may harbour a small-cyst population.

In contrast to the narrowing of the shape of the cysts in this *Blatta*, the cysts from the other two nymphal *Blatta* were actually slightly fatter than those from their respective donors. The decline in the size of these cysts was less than in those from the corresponding nymphal *Periplaneta*, but it is striking that in this test, just as in tests 2 and 3, the cysts from the recipient were, without exception, smaller in average size than those from the donor. There is no obvious reason for this: the temperature of the environment was constant: species, sex, age and diet were varied, but there is no evidence that any of these was the dominant factor.

The humidity of the environment may play a part in determining the size of the cysts in the host's faeces. On one hand, all the donors were kept routinely in pots, the floors of which were covered with wet filter-paper in order to keep their faeces moist and fresh, whereas all the recipients were kept in pots without wet filter-paper and the relative humidity of their environment was presumably lower; but on the other hand, although the main stock-tanks of *Blatta* were much drier than the pots, these cockroaches produced large rotund cysts.

The flora in the cockroach's hind-gut have not been investigated in these tests. Indeed, it was assumed that enough faeces were fed to each recipient to ensure that its 'daughter population' of bacteria would be identical with that of the donor.

A 'social factor' suggests itself, since those cockroaches in the stock-tanks were so crowded that they may have had some physiological effect on each other, and each was re-infecting itself repeatedly by eating faecal material from others. In contrast, the recipient cockroaches used in these tests were kept in isolation. However, it was shown in §(*b*) of this Part that the cysts in the faeces of a cockroach kept in isolation maintain a constant size and shape over a long period of time. Nor is there any evidence to show that there is an increase in size when the host moults.

Nevertheless, these five nymphs were used to discover if there is a 'social factor' and to test the effect of humidity. The two nymphs which had been infected with faeces from *Periplaneta* I were each fed some faeces from the other. This was repeated 5 days later and the nymphs kept for 3 weeks under the same conditions as before. As a control, each of the two nymphs, which had been infected with

faeces from the *Blatta*, was fed with some of its own faeces on two occasions and kept for a further 3 weeks. Three samples of cysts from each of the four nymphs were measured; the figures are presented in Table 3 *a* and *b*.

Table 3*a*. *Change of size after treatments*

	Average lengths (μ) after treatments		
	Periplaneta I	*Periplaneta* II	*Blatta*
Recipient *Blatta*	60·0 ± 1·6	48·2 ± 1·8	67·0
Difference between same before treatment	+ 4·3	+ 6·0	+ 3·2
Level of significance	Less than 1 %	Less than 1 %	—
Difference now between donor and recipient	− 5·2	− 24·9	− 2·3
Level of significance	Less than 1 %	Less than 0·1 %	—
Recipient *Periplaneta*	56·0	—	55·9 ± 2·2
Difference between same before treatment	+ 1·2	—	− 5·2
Level of significance	—	—	Less than 1 %
Difference now between donor and recipient	− 9·2	—	− 13·4
Level of significance	—	—	Less than 0·1 %

Table 3*b*. *Average ratio of length to breadth after treatment*

	Periplaneta I	*Periplaneta* II	*Blatta*
Recipient *Blatta*	1·30 ± 0·01	1·74 ± 0·19	1·43
Difference between same before treatment	− 0·02	− 0·04	− 0·08
Level of significance	More than 5 %	More than 5 %	—
Difference now between donor and recipient	− 0·06	+ 0·31	0
Level of significance	More than 5 %	Less than 0·1 %	—
Recipient *Periplaneta*	1·37	—	1·47 ± 0·03
Difference between same before treatment	+ 0·01	—	+ 0·12
Level of significance	—	—	Less than 5 %
Difference now between donor and recipient	+ 0·01	—	+ 0·04
Level of significance	—	—	More than 5 %

Cysts from the *Blatta* of the first pair had made a significant increase in size; those from the *Periplaneta* of the second pair had, in contrast, made a significant decrease in size. The shapes had remained more or less constant in both cases. The other nymphs in each pair yielded insufficient cysts for strict analysis, but in both cases there had been small increases in size and no very big change in shape.

The *Blatta* nymph which had been infected with faeces from *Periplaneta* II was used to test the effect of humidity. It was twice fed with some of its own faeces, and maintained for 3 weeks at a much higher humidity than before. Its pot was

put within a larger muslin-covered jar, the floor of which was covered with water. After 3 weeks, three samples of cysts were measured—see Table 3*a* and *b*. The cysts had increased significantly in size, being no longer as minute as before; nevertheless, they were still much smaller than those of the donor. There had been no significant change in the shape of the cysts.

The general inference from these tests is that the changes in size were not related to the conditions of the tests, since there was sometimes an increase, sometimes a decrease. Secondly, neither eating faeces from other cockroaches nor the degree of humidity can be of the order of importance required to account for the general phenomenon of the cysts from recipients being smaller than those from donors, for the negative reason that, if either were, the changes would have been more dramatic. This would have warranted tests on a larger scale. The relatively small changes in the sizes of the cysts from all nymphs may have been merely part of a general slow drift towards those which are typical for the species of hosts.

C. *Temperature*

Three adult *Periplaneta* were used in a study of the effect of the temperature of the host's environment on the average size and shape of the *Nyctotherus* cysts. A few *Blatta* and *Blaberus* were tried too, but failed to yield faeces regularly and soon died. All three *Periplaneta* were heavily infected and all had been reared at 25° C. The temperatures used in these experiments were 30° C and room temperature (approximately 20° C.). The cockroaches were kept routinely in pots, the floors of which were lined with wet filter-paper; and given the routine diet of apple, lettuce, horse-heart meat and rat-cake.

Throughout the experiment, all the faeces found in the pots were collected each day, and a sample of the cysts was measured. On some days, especially at the lower temperature, no faeces were produced: this was a corollary of the comparative lethargy of the cockroaches.

The results are presented graphically in Fig. 4A–C. Six samples were taken from the first cockroach (Fig. 4A) before the start of the experiment. It yielded faeces on all but one of the subsequent eleven days when it was kept at 30° C. Thereafter it was kept at 20° C. and produced faeces less frequently. It died 10 days after the last collection of faeces.

The average length of the cysts increased by about 7 μ (10%) when the cockroach was put at 30° C. and maintained the larger size. However, this slowly declined during the subsequent period at 20° C. The average ratio of length to breadth fluctuated, but in a fashion which seemed to be unrelated to the temperature.

The two other cockroaches were each put for two short periods at 30° C., separated and then kept for a period at 20° C. The cysts from one (Fig. 4B) actually decreased in size during both periods at 30° C. But when put at 20° C., there was a delayed rise, followed by a slow fall. The ratio of length to breadth also fluctuated, but in this case, it seemed to keep in phase with the fluctuations in size. In other words, the change in the average dimensions of the cysts was in the length; the breadth remained more or less constant.

The reason for there being relatively few points in Fig. 4B is that the cockroach defaecated infrequently at 20° C., and when put at 30° C. for the first time (but not the second), there was a sharp decline in the numbers of the cysts in the faeces, which made it necessary to amalgamate the measurements made on successive days into one sample and point.

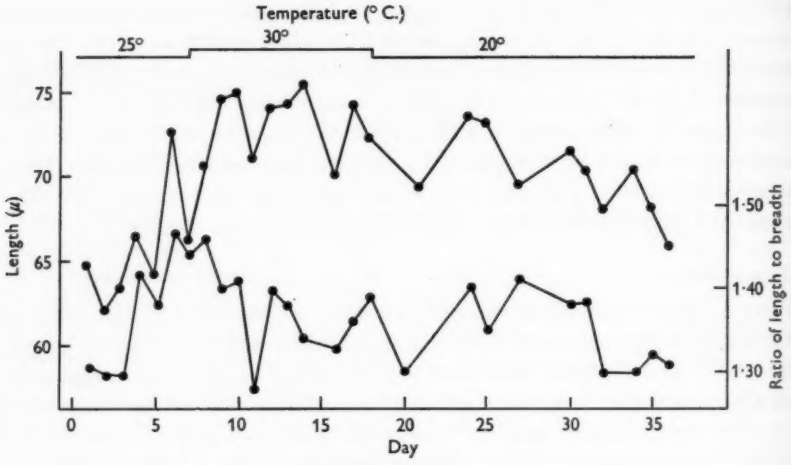

Fig. 4A

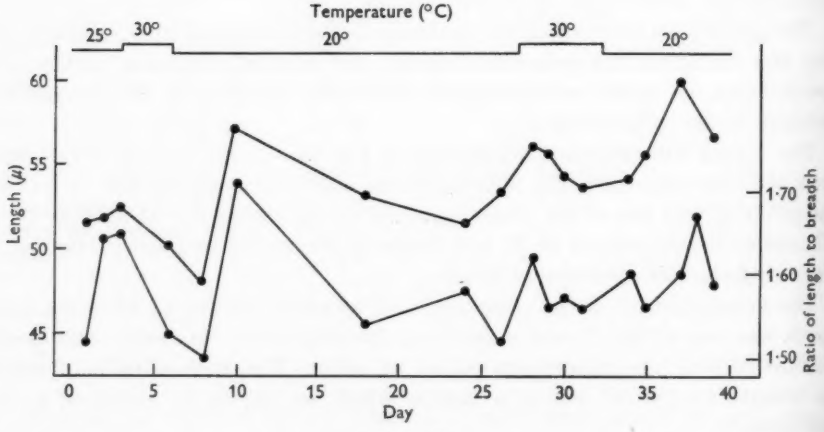

Fig. 4B

Fig. 4. A, B and C. The effect of fluctuations in the environmental temperatures of three specimens of *Periplaneta americana* on the daily average length (upper chart) and shape (lower chart) of the cysts of *Nyctotherus ovalis* in their faeces.

The two functions also fluctuated in phase in the case of the third cockroach (Fig. 4C). These cysts were like those from the first, but unlike those from the second cockroach, in showing a rapid response to the increases in temperature. In

both B and C the increase in the average length of the cysts was about 10 %: but in neither case did the second period at the higher temperature result in an increased step-up, although it involved a jump of 10° C. as opposed to 5° C. in the first instance.

The general conclusion that may be drawn from these observations is that an increase in the environmental temperature of the cockroach induced an increase in the average size of the cysts, and this seemed to be maintained for at least the short periods it was tried. However, it was not maintained during subsequent periods at lower temperatures.

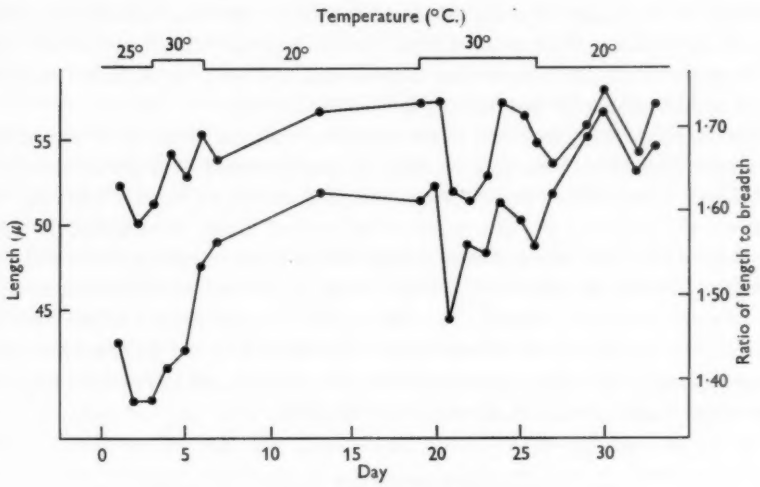

Fig. 4C

For legend see previous page.

The effect may have been direct on the ciliates: at the higher temperature the trophozoites may have remained the same average size but they may have encysted before achieving the usual reduction in size by repeated division, or the 10 % increase in the size of the cysts may have been merely part of a general increase in the average size of cysts and trophozoites, or, there may have been selection for large size. Alternatively, the effect may have been indirect on the ciliate: the flora may have changed and a new equilibrium achieved.

A clue which helps in the choice between these four possibilities is the persistence of the increase for a period after the hosts were put at the lower temperature. This would seem to rule out the first two possibilities, and seem to favour the third or fourth.

The general rule is for poikilothermic animals, in contrast to homiotherms, to be larger in a warmer environment than in a cold (see, for instance, Allee, Emerson, Park, Park & Schmidt, 1949). Much of the work on the effect of temperature on Protozoa deals only with the effect on the frequency of division, and no information is given on the change in size, if any, of the organisms. Simitch &

Petrovitch (1951) did describe cultures of *Entamoeba muris* at 22–23° C. and noted that the trophozoites, but not the cysts, were larger in the cultures than in those obtained directly from mice. But it is possible that this was an effect of a constituent of the culture medium, although no starch was used. However, Lom (1956) noted that cultures of *N. ovalis* and *N. cordiformis* maintained at room temperature, i.e. one normal to the hosts in nature, contained trophozoites of normal size, and cysts which were abnormally small. Thus, there is no evidence favouring the interpretation that high temperature itself selects for a larger size of cyst or induces a special phenotype.

The fourth possibility may obtain if the high temperature induced a serious alteration in the balanced composition of the flora—the near-elimination of one or more of the bacteria. This is not difficult to envisage and does not conflict with the idea that the form of *Nyctotherus*, trophozoite and cyst, is unstable and closely related to the quality of the flora in the host's hind-gut.

McConnachie (1954) reported some apposite work on cultures of two strains of *Entamoeba invadens*. One was growing in the presence of a *Bacterium coli* and 'organism *x*', the other (derived from the first) in the presence of *Bacillus megatherium* and 'organism *x*'. The cysts of the second strain were about 16 % larger than those of the first when grown in inspissated horse serum covered with Ringer eggwhite + starch, and about 20 % larger when grown in liquid horse serum diluted with Ringer's solution + starch. The differences were attributed to the influence of the two different species of concomitant bacteria and to the different composition of the two media. The effects were indirect: the smaller cysts were from populations of amoebae which grew and divided more rapidly.

III. THE STRUCTURE OF THE CYST WALL

(*a*) *Introduction*

Protozoan cysts are recognized as the resistant stage in the life-cycle. In both free-living and parasitic Protozoa, the wall of the cyst must protect the organism and yet, when the cyst returns to an environment favourable to the trophozoite, allow the entry of the factor stimulating excystation.

The chemical nature of the cyst wall was studied by Kofoid, McNeil & Kopac (1931) in four parasites of man: '*Entamoeba histolytica, Endolimax nana, Councilmania lafleuri, C. dissimilis* and *Giardia lamblia*' (both *C. lafleuri* and *C. dissimilis* are synonyms of *Entamoeba coli*). By studying the reactions in acids, alkalis and enzymes and using two histochemical tests for protein, they reached the conclusion, by process of elimination, that the cyst wall is a keratin. They did not have enough material, however, to carry out a chemical test for unoxidized sulphur or high cystine content: apparently, there were no satisfactory histochemical tests for keratin known at that time.

Lucas (1927, 1928) gave a clear description of the cysts of *Nyctotherus ovalis*. She saw that the wall consists of four layers: layer 1 is a thin, hyaline and colourless outer shell of even thickness; layer 2 is pale yellow and thin except in the knob formed at one end of the cyst; layer 3 is also yellow but, in contrast to layer 2, is

thick in the wall but relatively thin in the knob; layer 4 appears to be colourless, but is too thin for this to be certain. She was not explicit on this point, but layer 4 seems to me to line all the cyst wall except the inside of the knob. Pai & Wang (1948) stated that Lucas's description was incorrect in that layers 2 and 3 of the knob are not continuous with those of the wall, but instead the knob forms a definite plug. I think they were incorrect, and that the 'plug' is an artifact caused by viewing the cyst when it is not in full profile: the line or zone of change in the relative thicknesses of layers 2 and 3 round the base of the knob then gives the appearance of a definite break. Neither Lucas nor Pai & Wang described the chemical constitution of the cyst wall.

Lapage (1935) used 'Milton',* chlorine and NaOH to study the mechanism of the second ecdysis in *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus* spp. He found that 'Milton' caused ecdysis and subsequent dissolution of the sheath, but neither chlorine nor NaOH alone was effective. Chlorine did, however, cause (1) a blistering of the outer layer of the sheath exclusive of the cap, (2) a thinning of the sheath exclusive of the cap, (3) a distension of the whole sheath by internal pressure. If the treatment with chlorine was followed by NaOH, the sheath then dissolved.

In the following series of observations, acids, alkalis, chlorine and histochemical tests were used to differentiate between and identify the four layers in the cysts of *Nyctotherus ovalis*.

(*b*) *Material and methods*

The cysts used in the following tests were the largest type, derived from *Blatta* or from *Periplaneta*. Very few observations were made on the small type from *Blattella* or *Blaberus*, because the absolute thickness of the different layers in the knob and wall of these cysts is less, and it is less easy therefore to distinguish their various reactions to the stains and agents.

The methods followed in the histochemical tests were those set out by Pearse (1953) and are not here given in detail. In some cases a simplified method was used since permanent preparations were not desired. Tests using inorganic acids, alkalis, etc., were done on the stage of a microscope in order that the reactions could be watched, in most cases, under an oil-immersion objective. Cysts were picked out of faeces diluted with water and put in a drop of distilled water on a slide. A coverslip was sealed down along the edges parallel to the long sides of the slide. Successive drops of the agent were put at one free edge of the coverslip and drawn through with a piece of filter paper applied to the other free edge. When different agents were used in succession, they were drawn through in alternate directions.

(*c*) *General nature of the cyst wall*

Histochemical tests were made for carbohydrate, protein and fat.

Lugol's iodine was used as a test for carbohydrate. It stained the cyst wall brown, which indicated glycogen. However, cysts incubated in salivary amylase for 1 hr. at 37° C. still gave a positive reaction, showing the stained substance to

* A proprietary antiseptic containing sodium hypochlorite 1 %, sodium chloride 16·50 %.

be protein or amyloid. The latter was eliminated by testing the cysts in dahlia: they did not stain pink, but instead a pale blue.

Tryosine was indicated by a positive reaction to Millon's reagent. Layers 2 and 3 were tinged with an orange colour, but layers 1 and 4 seemed to take up little, if any, stain. Possibly these two layers were also tinged with orange, but because they are much thinner than layers 2 and 3, the colour was not apparent. Arginine was tested for, using Baker's modification of the Sakaguchi reaction. Layers 2 and 3 again gave a positive reaction; that of layers 1 and 4 was again doubtful.

Fat was shown to be absent by boiling the cysts in chloroform for eight hours: all layers remained intact. Secondly, negative reactions were given to Sudan Black B and Sudan IV stains.

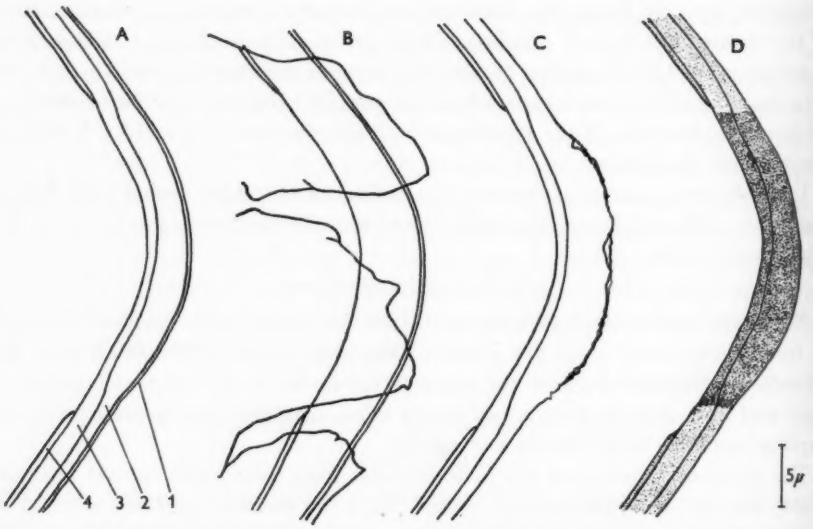

Fig. 5. A–D. The structure of the cyst wall of *Nyctotherus ovalis*. A, Fresh preparation, showing the knob and the neighbouring part of the wall proper. The four layers are designated by the numbers referred to in the text. B, After treatment with concentrated sulphuric acid. C, After treatment with chlorine water. D, Histochemical test for sulphydryl groups, after preliminary reduction of the SS bonds. The stippling indicates the intensity of the coloration.

(*d*) *Effect of concentrated acids in the cold*

Treatment with sulphuric acid revealed a membrane outside layer 1: it was lifted off as a partly fragmented sheet, but not dissolved (see Fig. 5B). This membrane was invisible in untreated cysts, and was not seen in the histochemical tests.

Layers 1 and 4 seemed to be unaffected by sulphuric, hydrochloric and nitric acids. Layers 2 and 3 swelled and amalgamated in sulphuric acid, swelled but did not amalgamate in hydrochloric acid, and layer 2 but not layer 3 swelled in nitric acid. The stress caused by the dissimilar response of these two layers to nitric acid caused a distinct crack to appear in layer 2 round the base of the knob. The cyto-

plasm was killed in every case, therefore all layers were permeable to the acids even though some appeared to be unaffected.

The effect of glacial acetic acid was to swell and amalgamate layers 2 and 3. There was a slight corrosion of layer 2 in the knob at its outer edge. The membrane, and layers 1 and 4 appeared to be unaffected.

(*e*) *Effect of 'Milton' and its constituents*

The crude effect of pure 'Milton' was to dissolve the knob on the cyst. The first event was the appearance of a crack round the base of the knob after 30 sec. or less; this was usually accompanied by a sudden shrinkage of the protoplasm, but this was reversed in the next ½ to 1 min. After the crack had appeared, the knob showed obvious signs of swelling and, at the same time, dissolution. It was completely dissolved after 2–4 min. in pure 'Milton'.

This was the usual course of events, but sometimes the knob was pushed off violently after about 1 min., before it was fully dissolved. This seemed to be caused by a tongue of protoplasm, pushing at the inside of the knob. The internal pressure developed after the initial shrinkage of the protoplasm and was not usually so vigorous.

The events were seen in more detail if 10 % 'Milton' was used. The time taken for total dissolution of the knob was about 15 min. The swelling was first apparent after ½–1 min. and the knob then steadily fattened. Viewed from the side, it looked like a fat grey sausage or a crescent: it was stiffly arched outwards, being held in place by the external membrane and its edge was still within the edge of the hole it left in the cyst wall. The knob then quietly dissolved away, passing through a stage when it was a mere pale ghost. The external membrane was sometimes forced to rupture before this dissolution, either by the pressure of the arching knob, or possibly by the tongue of protoplasm which again pushed out after an initial shrinkage.

These stages could be seen just as clearly, and the whole process took a proportionately shorter time, if 50 % 'Milton' was used.

(1) *Effect of* NaOH

'Milton' is a mixture of about 1 g. NaOCl and 16·5 g. NaCl in 100 ml. of water. Since both Na and OH ions are formed in the mixture, the effect of pure NaOH was tried.

A 0·1 % solution induced a swelling in layer 2 in both the knob and the wall, but all other layers seemed to be unaffected. Layer 2 did not dissolve subsequently.

(2) *Effect of hypochlorite*

A solution of NaOCl was used which was old and of uncertain strength: it was probably rather less than 1 %. It produced a swelling and dissolution of the knob in about the same time as a 60 or 70 % solution of 'Milton'.

A 2 % solution of 'bleaching powder' (partly $CaOCl_2$) produced as rapid a dissolution as pure 'Milton'.

(3) *Effect of pure chlorine*

Water saturated with chlorine gas completely dissolved layer 1. The fate of the membrane was not observed but presumably it was dissolved too. Layer 2 swelled in the knob and close inspection showed that it was slightly corroded at its outer edge. Layer 2 in the wall and the whole of layers 3 and 4 seemed to be unaffected (see Fig. 5C).

The effect of chlorine was most clearly seen in a preparation of the cysts in a hanging-drop of liquid paraffin. The coverslip was inverted over a small glass gas-chamber, affixed to a slide, and chlorine gas pumped through. Layer 1 was seen to bubble out in pimples and mushrooms, and the fragments were carried away by the currents in the paraffin induced by the gas sweeping by. In water containing chlorine, layer 1 quietly disappeared: presumably the products of dissolution are water-soluble.

Saturated bromine or iodine water had no visible effect. There was, however, a concealed effect described in §5.

(4) *Effect of* NaOH *followed by chlorine*

The effect of NaOH alone has already been described. If it were followed by chlorine water, layer 1 disappeared as before; and layer 2, already slightly swollen in the NaOH, partly dissolved at the knob in the chlorine. The dissolution was not complete but was rather more apparent than in chlorine alone.

(5) *Effect of chlorine followed by* NaOH

Chlorine alone produced the same effect as before, but when followed by NaOH, there was a rapid dissolution of the whole knob and the rest of the cyst enlarged. The absolute size was greater and there was a great increase in the thickness of the wall of the cyst. It was difficult to be sure if the cyst wall then consisted of one or of two layers, but the clearest examples seen suggested that layer 3 was unchanged but layer 2 was about ten times thicker than before. Layer 4 could not be seen.

If saturated bromine water were substituted for chlorine, layers 2 and 3 in the knob swelled out into a ghost-like crescent but did not dissolve. These layers were unaffected in the wall and layer 1 was evident as a sheet, holding the crescent in place. Substitution of saturated iodine water produced a slighter effect, but the effect was greater than if NaOH were used alone.

(6) *Effect of chlorine followed by nitric acid*

Chlorine by itself produced the dissolution of layer 1 and the swelling of layer 2 in the knob. If chlorine were followed by treatment with concentrated nitric acid, there was a rapid swelling and the destruction of both layers in the knob, followed by swelling, distortion and final dissolution of part of the wall. There was finally one layer left; probably layer 3.

(7) *Effect of sulphuric acid, water, chlorine and* NaOH *in succession*

Sulphuric acid swelled and amalgamated layers 2 and 3, but when this was followed by water, the layers separated again. The whole cyst swelled and all layers were stretched, appearing to be thinner. If the cyst were next treated with chlorine, layer 1 dissolved and the fragments of the membrane disappeared; layer 3, and layer 2 in the wall, seemed to be unaffected; but layer 2 in the knob swelled out. There was now a crack round the base of the knob. Addition of NaOH produced a total dissolution of the cyst: the knob first dissolved rapidly away, then the wall and finally the remnants of the protoplasm.

An additional treatment with NaOH can be inserted between the periods in water and chlorine, but the changes in the chlorine were much the same as above, and the complete dissolution was delayed until the final (second) time in NaOH.

(*f*) *Effect of proteolytic enzymes*

Incubation overnight at 37° C. in a 1% suspension of pancreatic trypsin, buffered at pH 8, gave variable results. The least effect seen was a slight corrosion of layer 2 in the knob. In a few cysts, however, a greater response was shown: the membrane was still intact; layer 1 was intact except on the knob where it was wholly or partly corroded; layer 2 was much corroded in the knob, and in the wall was swollen and partly digested; layer 3 in the knob, like layer 2, was much corroded, but was broken in the wall in only one or two places; and layer 4 was like layer 3.

Similar preparations with pepsin or papain gave similar results, but the protoplasm inside the cyst was killed.

(*g*) *Tests for keratin*

The general nature of the reactions of the cysts to treatment with inorganic agents and proteolytic enzymes suggests that one or more layers consist of keratin. This hypothesis was tested first by treating a human hair with a succession of concentrated sulphuric acid, water, chlorine and NaOH: its response was similar to layers 2 and 3. The fibrous elements of the hair were revealed in the acid, and they were evidently tougher than the substance of the cyst wall because it was found necessary to repeat the succession two or three times before the whole hair dissolved.

Keratin was tested for histochemically, using the Chèvremont and Fréderic ferricyanide method for sulphydryl groups. Fresh cysts gave negative results. However, if they were left in 10% KCN or 10% thioglycolic acid overnight at room temperature, the SS bonds were reduced to SH and a positive reaction was obtained. Neither the membrane nor layer 1 could be seen, but both layers 2 and 3 of the knob stained a strong Prussian blue after 10 or 20 min. At this time, there was no perceptible colour in layers 2–4 of the wall, but after a further 10 or 20 min., they too became tinged with blue. The intensity of the colour never developed to the strong blue shown by the knob (see Fig. 5D).

(*h*) *Discussion*

The reaction of the various layers to the different agents, and the positive result in the histochemical test for keratin, indicate that the chief constituent of the cyst wall is keratin. The variations in the reactions showed that keratin exists in different forms in the different layers, and seemed to be least stable in the knob. Arranged in order of increasing stability, the parts of the cyst wall are as follows: layer 2 of the knob, layer 3 of the knob, layer 2 of the wall, layers 3 and 4 of the wall. Clearly, then, the most vulnerable part of the cyst wall is the knob, and it is therefore most likely that the excystment factor enters through this region.

Inspection of cysts recovered from the mid-gut, after they have been ingested by a cockroach, suggested that all layers were still intact. The experimental evidence described in the third paper of this series (Hoyte, 1961*b*) indicates, however, that there must have been an invisible effect; because the newly developed ciliate within the cyst is unable to emerge unless the cyst has first been exposed to the digestive trypsin of the cockroach.

This attack by trypsin must necessarily be preceded by a reduction of the SS bonds in the keratin. Thus the reducing conditions of the gut not only stimulate excystation but make emergence mechanically possible. The lifting of the knob as an intact trap-door is made possible by the line of weakness running round its base. Here the change occurs in the relative thickness of layers 2 and 3, and here will be most felt the stress set up by the differential effect of trypsin on these two layers.

The constitution of layer 4 is probably keratin also. That of layer 1 is uncertain. Both chlorine and concentrated nitric acid are oxidizing agents, but only the former seemed to affect it. In fact its dissolution in chlorine was the only positive reaction it made to any of the agents or histochemical tests that were tried. Possibly its function is to provide a shell against mechanical damage of the underlying keratinous layers.

Keratin occurs in many groups of animals. The classification adopted by Block & Bolling (1939) divides keratins into two groups: the hair–feather–horn–quill eukeratins and the skin–neurokeratin–baleen–spongin pseudokeratins. The former is much more resistant to enzymic digestion and contains histidine, lysine and arginine in molecular ratios of approximately 1:4:12; the latter are less resistant and contain other proportions of these amino acids. The resistance to proteolytic digestion depends, in part, on the integrity of the fibrillar structure and is lost when keratin is broken down mechanically or chemically.

There is insufficient evidence to show whether the keratin of *Nyctotherus* cysts is a eukeratin or not. The vulnerability of the 'unstable' keratin of the knob could be due to it being formed of a pseudokeratin or could reside in an unorganized state of a eukeratin. The resistance of the 'stable' keratin of the wall, on the other hand, is almost certainly that of a eukeratin: that of layer 3 being more stable or physically organized than that of layer 2. The synthetic processes leading to the two substances are quite distinct, and it is unlikely that they should exist side by side, and, furthermore, that one should give rise to a structure whose layers are continuous with

those produced by the other. The vulnerability of the knob is therefore probably due to a lack of organization of the keratin in layers 2 and 3 into fibres.

The primitive function of keratin seems to be the provision of mechanical protection, but it appears in a variety of structures in higher organisms. Even in lower organisms it is elaborated into supporting structures: the spongin of Porifera, and probably the sulphur-containing protein of the cytosquelette in the ciliates *Radiophyra* and *Metaradiophyra* described by de Puytorac (1951). Its properties make it ideal for the protection of the resistant stages of Protozoa. It is not the only material, however, which can be synthesized and formed into cyst-walls by the Protozoa: Sachs (1956) demonstrated that chitin is the chief constituent in those of *Pelomyxa illinoisensis*. The occurrence of keratin and chitin in the Protozoa suggests that the origins of the two processes of synthesization lie very far back in evolution.

IV. SUMMARY

The size and shape of the trophozoite of *Nyctotherus ovalis* in different species of cockroaches was found to vary considerably, and this was attributed chiefly to the presence or absence of large paraglycogen granules within the ciliate.

The size and shape of the cysts of *N. ovalis* which were passed in the faeces of the host on successive days were found to fluctuate around means which were more or less constant over a long period. The constants varied according to the species of the host: the largest and fattest cysts were found in the faeces from *Blatta*, the smallest and most slender from *Blattella*. Those from *Blaberus* were of the *Blattella*-type, but *Periplaneta* harboured populations which gave rise either to the *Blatta*-type of cysts or to the *Blattella*-type.

The constant size and shape of the cysts was not altered when the host moulted, but there was always a decrease in size and sometimes a change of shape when an infection was established in a new host. The cause of these changes is unknown. An increase in size was induced by an increase in environmental temperature, but the size declined during a subsequent period at lower temperatures. The work of other authors on changes in the sizes of protozoan cysts is discussed.

The cyst wall of *N. ovalis* consists of four separate layers plus an outer membrane. They all seemed to be proteinaceous in nature. Treatment with various inorganic reagents and proteolytic enzymes revealed that the knob is the most easily destroyed part of the wall. A histochemical test for keratin showed that this is the chief constituent of the wall. The keratin appeared to be least organized in the knob, and hence the knob is the least impermeable part of the wall.

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The protozoa occurring in the hind-gut of cockroaches

III. Factors affecting the dispersion of *Nyctotherus ovalis*

By H. M. D. HOYTE

*Molteno Institute, University of Cambridge**

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I. THE MODE OF TRANSMISSION OF *NYCTOTHERUS OVALIS* AND THE VIABILITY OF THE CYSTS

(*a*) *Introduction*

In experiments described in the first and second papers of this series (Hoyte, 1961*a*, *b*), infections of *Nyctotherus ovalis* were transferred from one cockroach to another by feeding cysts, or whole faeces, from the donor cockroach to the recipient. This was assumed to be the mode of transmission in nature; and this assumption was supported by the observations that (1) the very few trophozoites which do pass out in the faeces are quickly killed by the dry and aerobic conditions of the environment outside the host, and (2) cockroaches have no instinctive drive to eat fresh faeces, or to feed at the anus of an infected host as Goetsch (1936) observed in termites.

The first part of this paper describes some experimental evidence which suggests that the ingestion of cysts is the only mode of transmission, either natural or experimental. Also described are tests of the viability of the cysts under wet and dry conditions at temperatures ranging from $-18°$ to $37°$ C. and some observations on the resistance of the cysts to other extremes of environmental conditions.

* Present address: University of Queensland Veterinary School, Brisbane, Australia.

(*b*) *Tests of alternative modes of transmission*

Three alternative modes of transmission were tried: the feeding of trophozoites; the injection of trophozoites into the hind-gut, and the injection of cysts into the hind-gut. The trophozoites were those of *N. ovalis*, *Endamoeba blattae* and *Lophomonas striata* from heavily infected *Blatta orientalis*. The cysts of *Nyctotherus* were from the same source. The recipients were nymphal *Periplaneta americana* which had been reared free of Protozoa.

Series I

Ten specimens of *Blatta* were dissected and the contents of their hind-guts fed to twenty specimens of *Periplaneta*. The recipients had been starved of food and water for the previous week and were fed in the manner described in the section on 'cross-infection' (Part II). They were dissected in pairs at intervals of up to 24 hr. after feeding.

Living trophozoites were found only in the fore-gut. *Nyctotherus* was the most hardy, surviving for up to 7 hr. *Endamoeba* and *Lophomonas* were killed in 5 hr. or less. Cockroaches which were dissected 9 or more hr. after feeding were sometimes found to be harbouring one or more of the species of Protozoa in their hind-gut. These, however, were clearly trophozoites which had excysted very recently.

Series II

Several specimens of *Blatta* were dissected and the contents of their hind-guts diluted with a little 0·5 % NaCl solution. A braking-pipette was used to pick out at least twenty trophozoites of each of the three species of Protozoa and inject them through the anus of each of twenty recipients—as far forward as possible in the lumen of the hind-gut. The recipients were dissected in pairs at intervals of up to 12 hr. after the operation.

Living trophozoites of *Nyctotherus* were found up to 5 hr. after being injected; those of *Endamoeba* and *Lophomonas* survived for 2 hr. or less. Dead trophozoites rapidly degenerated and became indistinguishable from the general faecal matter.

Series III

Faeces from *Blatta* infected with *Nyctotherus* were diluted with saline and the cysts picked out with a braking-pipette. These were injected through the anus into the hind-gut of each of twenty recipient *Periplaneta*. The recipients, which were prevented from eating their own faeces, were dissected in pairs at intervals of up to 3 days.

The cysts could survive in the hind-gut unchanged for the full period of 3 days, but many were voided before this. None hatched.

(*c*) *The viability of the cysts*

Faeces from *Periplaneta*, *Blatta* and *Blaberus* infected with *Nyctotherus* were used in the following series of tests of the viability of the cysts.

Series I

Methods. The viability of the cysts was tested at four different temperatures, and under wet and under dry conditions of the environment. Infected faeces were stored in a 'deep freeze' (−18° C.), in a refrigerator (4° C.), at 25° C. and at 37° C. The methods of storing the faeces were as follows: those at −18° C. under wet conditions were placed in a small volume of water (the water froze to ice during the period of storage), whereas those kept at 4°, 25° and 37° C. were placed on pads of cotton-wool which were kept soaked with water during the period of storage. Faeces stored under dry conditions were kept in Petri dishes. Those faeces to be stored under dry conditions at −18° C. were first dried for one day at 25° C. and approximately 60% relative humidity before being put in the deep freeze. This step was omitted with the faeces stored at the other temperatures under dry conditions.

At intervals, the viability of the cysts was tested by feeding them to cockroaches. Those faeces which had been stored under dry conditions had to be re-wetted in order to make them palatable. The recipient cockroaches were usually adult male *Periplaneta americana* which had been kept without food or water for a week or more. These cockroaches are convenient because this species eats faeces more readily than others; adults will consume a larger quantity of faeces than nymphs, and males harbour smaller populations of Protozoa than females, hence simplifying the subsequent search for cysts and recently excysted trophozoites in the hind-gut. The dissection of the recipients was made 12 or more hours after feeding.

Results. Information on the survival times is presented in Fig. 1. These times are all maxima: many of the cysts were killed after lesser periods. The data obtained in some tests suggested that cysts originating from one or other of the species of cockroaches were more resistant to drying or to heat or to cold than those from others; but the results in all tests were not consistent in this respect.

The longest time that cysts survived was 21 weeks, at −18° C. in dry conditions: this was seven times longer than the survival at 25° C. under dry conditions—those pertaining to faeces dropped by infected cockroaches in the main stock-tanks. In nature, the longest time which cysts can be expected to survive is approximately 20 weeks: in wet conditions at a temperature between 4° and 25° C. This is a wide range of temperature and shows that, providing the faeces are kept moist, the temperatures which the host may encounter in nature have little differential effect on the viability of the cysts.

Series II

Infected faeces were freeze-dried and the viability of the cysts tested thereafter. The method used was basically that outlined by Annear (1956), but with two simplifications: (1) sterile precautions were not taken, (2) the faeces were not centrifuged while being freeze-dried, but instead were spread over the inner surfaces of the tubes before putting them in the freeze-dryer.

The faeces were thoroughly freeze-dried (6 hr. over phosphorous pentoxide at a

pressure of approximately 0·02 mm. Hg.) The sealed tubes were stored at room temperature. Before being fed to cockroaches, the faeces were re-wetted.

Twenty-two tests were made. Most were unsuccessful; but in a few up to 5% of the cysts survived. A few cysts were still viable after 1 month's storage (the longest period tried). The large type of cyst from *Blatta* appeared to be more resistant than the small type from *Periplaneta* or *Blaberus*: the latter rarely survived.

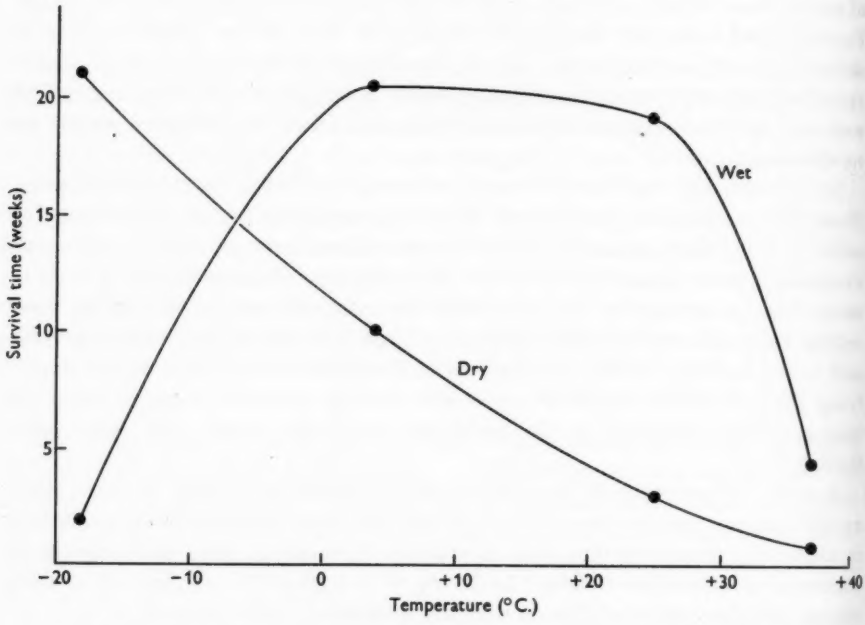

Fig. 1. The survival times of cysts of *Nyctotherus ovalis* in faeces kept under wet and dry conditions at −18°, 4°, 25° and 37° C.

A few tests were made to determine if the cysts were killed by the initial freeze-drying, or if they died during the subsequent storage. Faeces were freeze-dried in the normal way, but the tubes were not sealed and the faeces were immediately rewetted. The percentage of viable cysts was still very low, and this was true of cysts which were freeze-dried for only 2 hr. instead of the usual 6 hr.

Series III

Faeces were stored for up to 2 weeks at room temperature under wet and under dry conditions in pure oxygen, in carbon-dioxide and in nitrogen.

None of these conditions appeared to exert an adverse effect on the viability of the cysts.

(*d*) *Discussion*

The experiments described here suggest that the only way that infections can be transferred from one cockroach to another is by the ingestion of cysts in infected faeces or in the hind-gut of an infected cockroach which might be attacked and eaten by another. The death of ingested trophozoites is to be expected, but their death when injected into the hind-gut is of interest since a similar technique has been used to establish infections of other parasitic Protozoa in other animals. For example, Dobell (1938*b*) reports that Hlava (alias 'O. Uplavici') in 1887 succeeded in infecting cats with *Entamoeba histolytica* by an intrarectal injection of dysenteric stools (presumably containing amoebae) originating from human patients. Jones (1946) successfully carried out the same transfer, but used *E. histolytica* grown in culture. Another cross-infection between different species of hosts was achieved by Geiman (in Geiman & Ratcliffe, 1936). Frogs were injected intrarectally with cultures of *E. invadens*, originating from a snake, and this resulted in an infection of the frogs which was not accompanied by disease or tissue invasion. These successes suggest that either the Protozoa of the cockroach are less hardy in this respect, or that too small an inoculum was used, or that there was some other fault in the technique.

The survival time of the cysts of *Nyctotherus ovalis* was found by Lucas (1927) to be short. She stated that most of them degenerate in 8–14 days, but that some may survive for 17 days without showing any change in appearance. Lom (1956) found that culture-induced cysts kept in tap water remain viable for up to 87 days. These observations were made, presumably, on cysts kept at room temperature; the times were shown by my observations to be underestimates of those pertaining to cyst-survival in nature.

Most of the studies on survival of protozoan cysts under various conditions have been done on *Entamoeba histolytica*, because of its economic importance. An observation which is quite by the way is that of Meleney (in Andrews, 1942): he found that viable cysts of *E. histolytica* can be recovered from the intestines and droppings of cockroaches 48 hr. after being fed. Most tests have been made on the effects of various chemicals on the cysts. These include chlorine, iodine, ozone, silver nitrate, acetic acid, potassium permanganate and mercuric chloride.

The possible role of infected food-handlers was investigated by Spector & Buky (1934): they spread infected faeces on their fingers and thumbs and allowed the material to dry at room temperature: cysts rarely survived for more than 10 min. Reardon, Verder & Rees (1952) dried the cysts of *E. histolytica* and *E. coli* at a similar temperature and found that the former were irreversibly damaged, and that the cysts of *E. coli* also plasmolysed but this damage was reversible. In my experiments, the cysts of *Nyctotherus ovalis* survived for a much longer time under similar conditions: up to 23 days at 25° C.

Most of the studies of the effect of temperature on the viability of *E. histolytica* cysts have been based on observations on material kept under wet conditions. Simitch, Petrovitch & Chibalitch (1954) compared the viability of cysts kept in undiluted faeces with some kept in distilled water or in a physiological solution.

The time of survival at −6° C. was 1–4 days in the faeces, 1–3 days in water or solution. At 2–6° C., the survival time in faeces and in water was 20–40 days, but in physiological solution the cysts survived for 31–63 days. In all preparations, the survival time at 25° C. was about 3–4 days, and the time at 37° C. was about 1–2 days. An improvement in the viability of the cysts when washed free of faecal matter had also been noted by Yorke & Adams (1926): the survival time in faeces at 0° C. was 9 days, at 16–20° C. it was 7 days, whereas in a washed suspension at 0° C. it was 17 days, and at 16–20° C. it was 10 days. Dobell (1927) found a maximum survival time of 37 days at room temperature, and remarked that the best results were obtained with suspensions of cysts from cultures. Longer survival times have been obtained by Chang & Fair (1941) and Chang (1943) by storing the cysts at lower temperatures: the maximum time was 90 days at 0° C. Chang found that the data obtained over a range of temperature from 4–39° C. fitted a curve with the formula $t = 87{\cdot}2 \times 10^{-0{\cdot}04867T}$, where t = the time in days and T is the temperature in degrees Centigrade. Comparison of all these data with those from *Nyctotherus* show two points of difference: (1) the cysts of *Nyctotherus* survive for a longer time at all temperatures than those of *Entamoeba*, and (2) the shape of the curve plotted in Fig. 1 for the survival of cysts of *Nyctotherus* between 0° and 37° C. in wet conditions is convex whereas the curve for *Entamoeba* is concave.

The viability at sub-zero temperatures of *E. histolytica* cysts has been studied by Weinman & McAllister (1947), Halpern & Dolkart (1954) and Chang (1955). All authors found that subzero temperatures are detrimental to the viability: Chang observed a dehydration of the cysts which was caused by the crystallization of the water; Halpern & Dolkart found that a temperature of −15° C. for 24 hr. killed all the cysts, and Weinman & McAllister had only one successful result in twenty trials when they stored cysts at the same temperature for an unspecified time. The cysts of *Nyctotherus ovalis* remain viable for nearly 2 weeks at −18° C. under wet conditions but for 22 weeks under dry. In view of the dehydration of the cysts of *E. histolytica* observed by Chang, and the irreversibility of such damage which was observed by Reardon, Verder & Rees (1952), attempts to store the cysts of *E. histolytica* under dry conditions are likely to be as unsuccessful as under wet.

Storage of the cysts of *Nyctotherus* in preparations free of faecal matter may improve these survival times at subzero temperatures. The ability of the cysts of *Nyctotherus* to survive at −18° C. for up to 22 weeks, though superior to the ability of cysts of *E. histolytica*, is much inferior to the ability of *Trypanosoma*, *Leishmania*, *Trichomonas*, *Plasmodium* and pathogenic Metazoa studied by Weinman & McAllister (1947). These authors succeeded in storing these organisms at −15° and 70° C. for many months. Successful preservation of trypanosomes at low temperatures was also obtained by Reusse (1956) and Polge & Soltys (1957).

The effects of evacuation on the cysts of *Colpoda cucullus* were studied by Taylor & Strickland (1936). They found that slow evacuation did not kill the cysts, but rapid evacuation resulted in a mortality of about 40 %. Becquerel (1936) claimed that the cysts and resistant stages of some species of soil *Amoeba*, *Actinophrys*, *Paramecium*, rotifers and many algae can withstand evacuation and subsequent exposure to the temperatures of liquid helium and liquid nitrogen. The results

obtained from tests of the viability of freeze-dried cysts of *Nyctotherus ovalis* were poor: possibly alterations in the technique—slower evacuation, or the suspension of the cyst without faecal matter in a small volume of fluid—would give better results.

The brief tests of the viability of the cysts in oxygen, nitrogen and carbon dioxide could also be extended. The present results can be interpreted as showing that the bacteria of the faeces are able to provide the necessary micro-climate (Scholander, Claff & Sveinsson (1952) found that the oxygen consumption of resting cysts of *Bresslaua insidiatrix* is too small to be measured in a respirometer). However, the cysts contained in dry faeces survived as well as those in wet, and it is unlikely that bacteria are able to flourish in dry faeces. A second possibility is that there were small but sufficient quantities of impurities in the gases which satisfied the requirements of the encysted organisms. A third alternative is that the cysts are truly indifferent to their gaseous environment: metabolism may be suspended, or the wall may be impermeable to gasses and the metabolism of the organism may be a closed system.

II. EXCYSTATION OF *NYCTOTHERUS OVALIS*

(*a*) *Excystation* in vivo

(1) *Introduction*

Nyctotherus ovalis has been described from *Periplaneta americana*, *P. australasiae*, *Blatta orientalis*, *Blaberus giganteus* and *Blatella germanica*. The form of the trophozoite within these hosts, and of the cysts in their faeces, was shown in Hoyte (1961*b*) to vary between two extremes in size and shape, and this was in general related to the number of large storage granules within the trophozoite and cyst. An informative method of studying these variations would have been to culture *Nyctotherus* and to vary the culture medium; but attempts to do this were uniformly unsuccessful. An alternative method is by way of transferring an infection from one cockroach to another, and following its subsequent life in the new host. In nature, a cockroach picks up an infection by eating the cysts; these hatch in the mid-gut, and the trophozoites may then become established in the hind-gut. The experimental methods used in transferring infections under controlled conditions followed this same mode.

(2) *Cross-infection*

In nature, the small slender cyst seems to be restricted to *Blattella* and *Blaberus*, and may occur in *Periplaneta* but not in *Blatta*; whereas the large football-like cyst seems to be restricted to *Blatta*, and may occur in *Periplaneta*, but not in *Blattella* or *Blaberus*. This suggests that there is some degree of a real host-restriction and it is possible that, for example, cysts from *Blattella* are unable to excyst in *Blatta*, and vice versa.

Material. (1) Adult and large nymphal specimens of *Blatta orientalis*, *Blattella germanica* and *Periplaneta americana*; and small nymphal *Blaberus ginganteus*. All the cockroaches were either free of Protozoa or were only lightly infected.

(2) Fresh faeces, which contained cysts of *Nyctotherus*, collected from all four species of cockroaches.

Methods. Gunn (1940), Mellanby (1940), Cloudsley-Thompson (1953) and Harker (1956) have shown that the cockroach follows a diurnal rhythym of activity and feeding. It does not, therefore, immediately consume a meal of infected faeces whenever they are set before it. In any case, the accumulation of faeces within a chamber holding a cockroach demonstrates that it normally has little inclination to eat faeces.

In order to make a cockroach hungry, it was isolated in a small glass pot without food or water for upwards of 1 week, at a temperature of 25° C. The method used in securing *Periplaneta*, for convenient and supervised feeding of faeces, was an adaptation of one used by Rendtorff (1953) in feeding infectious material to houseflies. V-shaped notches were cut in the edge of a sheet of thin stiff paper (an index card is suitable), each notch some 8 mm. wide and 20 mm. long, and the card was held horizontally in a clamp. The neck of a cockroach was slid into each notch so that its head was above the card and its body below, and the cockroach secured in this position by a thin strip of sticking-plaster across the open end of the notch. A healthy animal would cling to the underside of the card, and would eat rapidly when given moist faeces.

This method was also used in feeding faeces to *Blatta* and *Blattella*, but cockroaches of the former species often seemed more receptive if the card was held vertically, and those of the latter species may be handled more easily if lightly anaesthetized with chloroform or ether, from which they soon recover. *Blaberus*, because of the enlarged pronotum, cannot be fitted into these notches. It was secured by holding it upside-down on a piece of cork and pushing two entomological pins through the flanges of the pronotum one on each side of the neck. This cockroach tended to vomit when handled, but would usually consume the vomit and infected faeces if it had been starved.

After the cockroach had eaten a good quantity of infected faeces, it was put back in its pot and kept thereafter at 25° C., as before. No food was given, or water; and some 12 hr. later (or more in the case of *Blaberus*), it was killed and dissected. By this time, many of the viable cysts had hatched and the trophozoites were to be seen in the contents of the hind-gut. These trophozoites could be distinguished very easily from the native population, by their small size, clear cytoplasm and the absence of an anterior septum. Ideally, one or two were seen in the process of emerging from their cysts.

Results. Cysts in the faeces of *Blattella* were able to hatch in the gut of *Blatta*, *Periplaneta* or *Blaberus* quite as readily as in *Blattella*. Cysts in the faeces of the other cockroaches were equally catholic. Thus, there is no apparent restriction operating at this stage in the life-cycles of the different types of *Nyctotherus*.

(3) *Initiation of an infection*

This section describes experiments to find factors which might restrict the initiation and establishing of an infection in the new host. The following variables were tested: age of the new host, its food supply, the number of times it need eat in-

fected faeces, its state in relation to moulting, the temperature and the humidity of its environment, and its prior infection with another population of *Nyctotherus*.

A. *Age of the new host.* First-instar nymphal *Periplaneta americana* and *Blatta orientalis* were used in a series of tests of the ability of *Nyctotherus* to excyst and establish itself in a very young host. These cockroaches are too small to be handled conveniently: it was considered impracticable to secure them and feed infected faeces to them directly. In the first series of tests, pairs of nymphs, which had hatched within the previous 24 hr. from sterile egg-cases, were confined in 10 cm. lengths of 6 mm. bore pyrex glass tubing, lightly plugged at each end with cotton-wool. Fresh infected faeces were put on the inside of one of the plugs and the plug made to act as a wick, to keep the faeces moist and reasonably palatable, by resting that end on the bottom of a Petri dish containing a little water, and supporting the other end on the edge of the dish. Thus, the infected faeces were the only available food.

The nymphs were taken out after an interval of at least 4 days; their hind-guts were dissected out and the contents examined. Many of the nymphs evidently had not eaten faeces; some had eaten, but their intestines contained no Protozoa; but others were found harbouring *Nyctotherus* after eating faeces from *Periplaneta americana*, *Blatta orientalis*, *Blaberus giganteus* or *Blattella germanica*. Thus, these very young nymphs are as capable of becoming infected with *Nyctotherus* as are adults and older nymphs.

The minimum interval of 4 days was long enough for an infection to be initiated, but of course a higher proportion of nymphs were found with *Nyctotherus* if a longer period was allowed to elapse. The infected faeces from *Periplaneta* and *Blatta* could also set up infections with *Endamoeba blattae* and *Monocercomonoides orthopterorum* within 4 or 5 days.

Similar results were obtained with both species of recipient nymphs.

B. *Availability of food.* In the second series of tests, groups of nymphal *Periplaneta americana*, which had hatched within the previous 24 hr. from sterile egg-cases, were confined in $3\frac{1}{2} \times 3$ in. round glass pots with infected adults and given a supply of apple, horse-heart meat and rat-cake. Thus, the nymphs were presented with the choice of making their first meals of infected faeces or of first eating the 'good' food.

Four adults were used, each infected with *Nyctotherus*, *Lophomonas striata* and *Monocercomonoides*, and some had *Lophomonas blattarum* as well. At least 1 week was allowed to elapse before any nymphs were removed from the pots, killed and examined: further batches were examined at increasing intervals.

The shortest interval in which the nymphs were found to develop an infection with any of the Protozoa was 3 weeks. Some remained free of infection, in spite of the crowded conditions in the pots, for as long as $6\frac{1}{2}$ weeks. *Monocercomonoides* appeared most frequently in the nymphs; *Nyctotherus* less so, *Lophomonas striata* was seen in very few nymphs and *L. blattarum* was never seen.

These observations demonstrated that the initiation of an infection with these Protozoa is delayed until cysts are ingested accidentally: there is no instinctive drive to eat faeces.

The relative frequency of the different Protozoa appearing in the nymphs may be construed merely as a rough indication of the relative concentrations of the different cysts in the faeces from the infected adults.

C. *Infection after one meal of infected faeces.* Certain data suggested that the cockroach must make a second meal of infected faeces before an infection with *Nyctotherus* is established in its hind-gut. This suggestion was supported by the observation of Hegner (1927*a*), who found that guinea-pigs do not necessarily become infected with *Iodamoeba williamsi*, although excystation itself was successful. Two clear examples of this phenomenon were mentioned in the second paper of this series, in connexion with the sizes of cysts: the specimen of *P. australasiae* was fed one lot of faeces containing viable cysts, but showed no sign of infection itself until it was fed faeces a second time; and the nymphal *P. americana* given only one meal of faeces from *Periplaneta* II failed to develop an infection, whereas the other nymphs which were used in the experiment were all given a second meal and infections were established in all.

This suggestion was disposed of briefly. A single meal of infected faeces was given to each of three large nymphal *Periplaneta* which had been reared free of Protozoa. The infected faeces originated from (*a*) a specimen of *Periplaneta* harbouring a large cyst population of *Nyctotherus*, (*b*) one harbouring a small cyst population, (*c*) a specimen of *Blaberus*, harbouring a small cyst population. Each nymph was put in a glass pot with a similar nymph which had not been fed faeces, and given an ample supply of food. All the nymphs were killed $2\frac{1}{2}$ weeks later and the contents of their hind-guts examined. Large numbers of *Nyctotherus* were found in each of those which had been fed infected faeces but in none of the controls. It may be assumed, therefore, that each infection was a genuine result of a single meal of infected faeces, and that none was the result of that meal reinforced by re-ingestion of the few cysts which failed to hatch during the first passage through the gut and which might still be viable, or of the cysts of the daughter population.

Lophomonas striata was established in nymph (*a*) and *Monocercomonoides* in nymph (*c*).

D. *Moulting in the new host. Nyctotherus* seemed to be unable to excyst in a cockroach which was about to moult. A few days prior to moulting, nymphal cockroaches start a period of self-starvation which lasts until the moult is complete and the new exoskeleton is hard. Numerous observations on nymphs in pre-moult showed that, although they might have been starved of food and water for a period of 1 or 2 weeks prior to entering this state of voluntary starvation, they could not be persuaded to eat even moist infected faeces. Some would, however, drink a little water from a pipette, and thus a few *Nyctotherus* cysts in suspension could be fed to them. Subsequent dissection showed the entire gut to be nearly empty of food, and the cysts, in a degenerate condition, stagnating in the fore-gut.

E. *Temperature of the environment.* Lucas (1928) showed that a temperature of 28° C. may be sufficient stimulus to hatch *Nyctotherus* cysts *in vitro*. She remarked that: 'The cyst would not be subjected to such an influence on entering the host, unless it be that the digestive processes occurring in the stomach, result in a

considerable evolution of heat.' This section describes tests of the ability of cysts to hatch *in vivo* at temperatures much below 28° C.

Three large nymphal *Periplaneta*, which had been reared free of gut Protozoa, were fed infected faeces from *Periplaneta*, *Blatta* and *Blaberus*. They were maintained thereafter at a temperature of 12° C., were given a supply of food, and were dissected on the seventh day after feeding. Newly excysted *Nyctotherus* were found in the hind-guts of all three hosts, but it was noted that some of the cysts from which the ciliates had emerged had not yet passed out of the gut. This implies that, at this temperature, the passage of food through the gut is very slow. A high proportion (about one-third) of the cysts originating from *Blaberus* had failed to start excystation, and one was found which was still in an early stage.

A fourth cockroach was fed faeces from *Periplaneta* and kept thereafter at 10° C. for 10 days. The cockroach was still alive but the meal was found to be still within the fore-gut: none had passed to the hind-gut. This temperature seems to be just below that which permits digestion.

Other *Periplaneta* were fed infected faeces and were maintained at 12° C. for shorter times, in order to find the minimum period for excystation to occur. Forty-four hours were found to be insufficient but 56 hr. were enough. The proportion of excysted and about-to-excyst *Nyctotherus* were about the same as in cockroaches kept at 25° C. for only 10 hr. This time of 56 hr. was shown to be a function of the host and not of the process of excystation itself by feeding infected faeces to some *Blattella*. In this smaller species, 24 hr. was long enough for hatching of most of the cysts to have occurred.

There seemed to be no obvious difference between the abilities of the cysts originating from the different species of cockroaches to excyst at these low temperatures. Some of the results did indicate, however, that the small-type cysts from *Blaberus* were slightly less successful; but this may have been a property of the faecal matter containing them since these donors were fed on lettuce whereas the others were fed apple, horse-heart meat, rat-cake and lettuce.

F. *Humidity of the environment.* The results of some preliminary experiments indicated that the Protozoa in the hind-gut are reduced in number when cockroaches are kept in a very dry environment, but flourish when the hosts are kept in humid surroundings. These results suggested that there may be marked difference in the ability of the Protozoa to establish infections in hosts kept under these conditions.

Faeces from an infected *Periplaneta* were divided into two portions and each half fed to a large nymphal *Periplaneta* which had been reared free of Protozoa. Similarly, faeces from a *Blatta* was given to two other *Periplaneta*. One of each pair of recipients was put in a desiccator, the other put on a platform inside a bell-jar inverted over a dish of water. Both lots were kept at 25° C. After 7 days, all were dissected and found to be infected with approximately the same numbers of *Nyctotherus*. In addition, smaller numbers of *Endamoeba* and *Lophomonas striata* were found in some of the recipients, but their distribution did not seem to be related to the relative humidities of the host's environment.

G. *Prior infection with another population of* Nyctotherus. The observations so

far recorded in this Section all relate to the fate of a population of *Nyctotherus*, introduced into a cockroach which had been free of Protozoa. The fate of the *Nyctotherus* introduced into a host which was already infected is of particular interest since it bears on what must be a recurrent event in any crowded stock of cockroaches of which a high proportion are already infected. It was necessary to choose an artificial situation, since it would be difficult to distinguish, say, an infection with typical *Blatta*-type of *Nyctotherus* imposed on the same type of infection. Therefore *Blattella*-type cysts from *Blattella* and *Blaberus* were fed to six adult and large nymphal *Blatta* which were known to be harbouring typical *Blatta*-type *Nyctotherus* (Hoyte, 1961*b*). Three days, 1 week and 3 weeks later, the *Blatta* were killed two at a time and the contents of their hind-guts examined. After only 3 days, the populations of *Nyctotherus* were still distinct: those which were indigenous were distinguished from the introduced *Nycotherus* by the relatively large size, the rounded anterior end, and the presence of large storage granules, at least in the region in front of the macro-nucleus; whereas the introduced *Nyctotherus* were small, had a pointed anterior end, lacked the large granules and still lacked the anterior septum. After 1, and 3 weeks, only the typical *Blatta*-type of *Nyctotherus* trophozoite and cyst were seen. This indicated that either the introduced population failed to become established and may have been supressed by direct competition with the indigenous population, or that it developed the characteristics of the indigenous population.

Some of the reverse experiments were unsuccessful. Large-type cysts were fed to three *Blattella* and these were killed 3 weeks later, after being kept at 25° C. in isolation on a diet of apple. *Nyctotherus* was absent from all three, although, as shown by the presence of cysts in their faeces, at least two had been infected before the start of the experiment. This procedure was repeated with four other *Blattella*, but these were fed infected faeces from *Blatta* followed, after an interval of 5 days, by a suspension of similar cysts in water. All but one of them were free of *Nyctotherus* after the same period of 3 weeks, although at least two had been infected before the start of the experiment. The fourth *Blattella* had a mixed infection: a few of both types of cysts were present. The trophozoites nearly all had large storage granules but most were small and had pointed anterior ends. These failures of the *Nyctotherus* from *Blatta* to establish itself in *Blattella* is supported by another observation. At one time, the stock-tank of *Blatta* became contaminated with a few *Blattella* and eight of these were examined. All were free of Protozoa, although some were infected with nematodes, indicating that faeces had been eaten.

Three small uninfected *Blaberus* nymphs were used in the next experiment. Two were fed on two occasions with infected *Blatta* faeces and on two occasions with infected *Blaberus* faeces, the meals being given in alternation. Similarly, the third nymph was fed with *Blatta* and *Blattella* faeces. All three nymphs were killed 3 weeks after the last meal and all three were found to be infected with the *Blatta*-type of *Nyctotherus*.

The conclusion which may be drawn from these observations, and those in Hoyte (1961*b*), is that either the *Blatta*-type of *Nyctotherus* competes with and excludes the *Blattella*-type, or that: (1) there is a basic type of *Nyctotherus*—the small

trophozoite, with a pointed anterior end and no large storage granules, which gives rise to a small slender cyst; (2) this form is modified, by association with a particular type of flora, into a larger trophozoite, with an anterior end which is swollen into a rounded shape, perhaps by the presence of the mass of large storage granules in front of the macronucleus, and which gives rise to a large and rotund cyst; (3) this flora is typical of the hind-gut of *Blatta* and may be found in *Periplaneta*; but this flora is typically absent from *Blattella* and *Blaberus*, and may be absent from *Periplaneta*.

(4) *The timing of the process of excystation*

Lucas (1928) described excystation in *Nyctotherus ovalis* and how it may be induced by maintaining the cyst at 28° C. in 0·5 % NaCl under a sealed coverslip. She also followed the process in cysts recovered from the mid-gut of a cockroach after it had been fed infected faeces or bread soaked in saline and infected faeces. She found, by feeding starved cockroaches with moist bread mixed with carmine, that it took about 16 hr. for food to pass through the hind-gut, although there was considerable variation from cockroach to cockroach. She found that excystation started in the mid-gut, about 11 hr. after the cysts were consumed by the host, and she estimated that the process took 6–8 hr.

Contrary evidence on the time required for the passage of food through the gut was given by Day & Powning (1949). They found that in a cockroach starved for 2 days, some food passes from the fore-gut into the mid-gut only 5 min. after feeding, and that food reaches the hind-gut after about 2 hr.

I found that cysts might reach the hind-gut and the process of excystation be completed in 3 hr., if the cysts were fed without faecal matter or food but as a suspension in water. If the cysts were fed in the form of infected faeces, newly hatched ciliates could be found in the hind-gut 8½ hr. after feeding. This was about three times longer than if the cysts were fed in a watery suspension with a minimum of faecal matter, but was only about half the time which Lucas found was taken under similar conditions. These times of 3 and 8½ hr. were found to be the minimum when the recipient cockroach was kept at 25° C. Lucas did not consider the temperature as a factor, but it is possible that in view of the results recorded in §3E above, the *in vivo* excystation she observed took as long as 16 hr. to complete because she maintained her cockroaches at a temperature 'not over 23° C.'.

An interval of 8½ hr. was the shortest that was found: one newly hatched *Nyctotherus* was found in one cockroach. Observations made at increasing intervals revealed larger numbers of newly hatched trophozoites in larger proportions of the recipients. These figures were obtained from large nymphal and adult *Periplaneta americana*, and were confirmed with *Blatta orientalis*. A few observations were made using adult *Blaberus giganteus* as recipients, but in this large cockroach it took about twice as long (18 hr.) for the cysts to reach the junction of the mid- and hind-guts and hatch. In the small cockroach, *Blattella germanica*, only 6½ hr. were needed for excystation to be completed. The process of excystation, by re-orientation, redifferentiation and final emergence, through the hole left in the cyst wall when the knob lifts, was described by Lucas (1928). Cysts in various stages of this process could be found throughout the length of the gut if a starved cockroach,

which had been fed some heavily infected faeces and kept thereafter at 25° C., was dissected 9 or more hr. later. It was noticeable that a minority of cysts failed to hatch. It is possible that some of these were cysts which were not fully matured before being ingested, but this is unlikely to be the reason in every case because, in faeces which were one or more days old, there was a similar minority of cysts which failed to hatch. Some of the failures seemed to be due to degenerative changes which occurred in some of those cysts which were forced to wait in the fore-gut while others made the passage of the mid-gut.

Although I found that the total time needed for excystation was shorter than that suggested by Lucas, I was unable to get cysts recovered from the mid-gut to complete *in vitro* excystation in the average time of 6–8 hr. that she found possible. She dissected out the whole contents of the mid-gut of a cockroach which had ingested infected faeces from 10 to 20 hr. beforehand, placed it on a slide, diluted it with 0·5 % NaCl and sealed the coverslip down. I repeated this operation, using material ingested 8–16 hr. beforehand by *Periplaneta* and *Blatta*, and kept the preparations at 25° C. About 24 hr. were needed for the completion of excystation, but only about 12 hr. if the cysts were already at some intermediate stage.

Cysts which showed no signs of excystation were picked out of the diluted mid-gut contents with a braking-pipette, put in a drop of saline on a slide and the coverslip sealed down. These cysts (almost entirely free of mid-gut contents) were able to hatch but took between 24 and 48 hr. to do so.

Another observation was that those cysts recovered from the mid-gut less than 11 hr. after feeding, failed to complete excystation. This in spite of some being recovered and included in the preparation when part-way through the process. They could get no further than completing the reorientation and redifferentiation, and they appeared to be unable to lift the knob and emerge.

These data indicate that: (1) the process of excystation is triggered off when the cyst reaches the mid-gut, but at this time there is no visible change; (2) the process may then proceed through the stages of reorientation and redifferentiation whether the cyst is in contact with mid-gut contents or not, but if the former, it proceeds faster, (3) the ciliate is unable to break out of the cyst unless it has been within the gut (fore- and mid-) for 11 hr. or more.

Investigations of the factor which triggers off the process are described on later pages. The final lifting of the knob is probably partly brought about by the movements of the ciliate within the cyst. The evidence suggests, however, that the knob must first be weakened sufficiently by the action of the host's digestive enzymes.

(5) *Excystation in hosts other than cockroaches*

Nyctotherus ovalis has been recorded from the cockroaches *Periplaneta americana*, *P. australasiae*, *Blatta orientalis*, *Blattella germanica* and I have found this ciliate in *Blaberus giganteus*. Stein (1867) found it in the mole-cricket *Gryllotalpa vulgaris* [= *G. gryllotalpa*], and Pai & Wang (1948) in *Periplaneta americana*, *Gryllotalpa vulgaris* and *Julus* sp. of China. Other species of *Nyctotherus* have been found in other insects and in Amphibia. However, the authors of these records did not ascertain whether the *Nyctotherus* they described, often differing only in

trivial ways from others of the same genus recorded in other hosts, were able to infect the other hosts. What is particularly suggestive is that the different forms of *N. ovalis* which are typical of the different species of cockroaches, are able to excyst and establish an infection in any of the cockroaches. Once established, there may be a change in the form which is of the same order of magnitude as the differences between some species of *Nyctotherus*. A number of different insects, a myriapod and a frog were tested to see if *Nyctotherus* of the cockroach would excyst and survive in them.

Two members of the order Orthoptera were tested. Seven adult and large nymphal specimens of *Schistocerca gregaria*, the desert locust, were fed infected faeces from *Periplaneta*, *Blatta* and *Blaberus*. They usually ate readily; and they were kept thereafter at room temperature, in large glass pots, the floors of which were lined with wet filter-paper. Their faeces were collected and the condition of the *Nyctotherus* cysts examined. Up to 15 % of the cysts of the large type (from *Periplaneta* and *Blatta*) and of the small type (from *Periplaneta*) had hatched or the ciliates were in the process of emerging from the cysts. A much smaller proportion (less than 1 %) of the small type from *Blaberus* succeeded in hatching. However, like the other cysts, about half had reorientated (the macronucleus had migrated to the knob end). Of the remaining cysts, some were degenerate, and in some of these the knob had lifted; but many were quite unaffected.

Dissection of locusts, which had eaten and passed these cysts, failed to reveal any ciliates in its hind-gut. It seemed that the passage of food through the gut was so rapid that the newly excysted *Nyctotherus* were swept out.

In attempts to increase the proportion of cysts which completed the process, the faeces from the locust were fed again, i.e. the cysts were made to pass through the gut twice. This merely increased the proportion which were degenerate. Ligating the abdomen of the locust as far to the rear as possible, so that it was unable to defaecate and the cysts were thus exposed to a prolonged treatment, also merely increased the proportion which were degenerate. Similar unsuccessful results were obtained when the faeces from the locust were put on a slide, the coverslip sealed and the preparation kept at 28° C. after being made up with (*a*) distilled water, or (*b*) 0·5 % NaCl, or (*c*) M/15 phosphate buffer at pH 8 + a 0·2 % suspension of trypsin, or (*d*) buffer alone.

In all the locust faeces which were examined, only one *Endamoeba blattae* and one *Lophomonas striata* had excysted—less than 1 % of the ingested cysts.

Four adult and large nymphal *Dixippus morosus*, the stick insect, were fed the same variety of faeces that the locusts received. They were dissected on the following day and cysts were found throughout the gut. None showed any signs of hatching and few any signs of degeneration.

Similar experiments were carried out using adult and large nymphal *Julus terrestis*, a millipede, adult *Tenebrio molitor*, a flour beetle, adult *Calliphora erythrocephala*, a blow-fly, and large larval *Pieris brassicae*, the cabbage-white butterfly. Similar negative results were had with these animals. Two adult female *Rana temporaria*, the common frog, were fed large quantities of infected faeces, but no cysts were stimulated to hatch and a few appeared to have degenerated.

(*b*) *Excystation* in vitro

In vitro excystation of *Nyctotherus ovalis* may be studied in two ways. One method is to simulate the events in the gut of the cockroach, the other is to repeat and define the unexpected results of Lucas (1928), who induced excystation merely by maintaining the cysts at 28° C.

(1) *Simulation of events in the gut*

A. *Cysts recovered from the mid-gut.* The results obtained when cysts were fed to cockroaches, recovered from the mid-gut, diluted with 0·5% NaCl and made to finish the process of hatching *in vitro* have been described (p. 478).

A more careful preparation was tried to see if the completion of the process could be speeded up. Starved cockroaches were fed infected faeces, killed 4½–13 hr. after feeding, the contents of each mid-gut dissected out on to a slide, diluted with M/15, pH 6·2 phosphate buffer and sealed under a coverslip. (The pH of the buffer is approximately that of the mid-gut contents as found by Wigglesworth, 1927.) The preparation was kept at 28° C. for up to 24 hr. Eleven trials were made and five were successful. In the other six, excystation was never started, or had started but the cysts degenerated. Probably the factor responsible for the failure to excyst was overgrowth by bacteria which produced an unfavourable environment.

The addition of this buffer had the effect of speeding up the completion of excystation: it occurred 7–10 hr. after making up the preparation. Although this is two or three times faster than that taken when I used 0·5% NaCl instead of buffer, it is no faster than the time Lucas found possible using saline; nor does it approach the rapidity of *in vivo* excystation. The preparations were also unsatisfactory in that no positive results were got with cysts recovered less than 10½ hr. after being fed to a cockroach.

B. *Injection of cysts into the excised mid-gut.* In this series, the passage through the fore-gut was assumed to be unnecessary for excystation and was therefore omitted. For each experiment, an adult female *Periplaneta* was killed, its abdomen opened and its gut gently pulled out. The mid-gut was ligated at the point of juncture with the hind-gut and the gut cut through just posterior to this. The gizzard was also cut, transversely, and the whole mid-gut placed in a solid watch-glass containing about 10 c.c. of insect-Ringer solution.

Faeces containing *Nyctotherus* cysts were smeared on a slide in 0·5% NaCl solution and the cysts picked out with a braking-pipette. The slender capillary tubing was then thrust into the lumen of the mid-gut for several millimetres, through the centre of the remaining part of the gizzard, and the cysts expelled. This was repeated several times until a dozen or more cysts lay within the mid-gut The remains of the gizzard made a convenient lug by which to hold the preparation while inserting and withdrawing the pipette, and the cysts were prevented from leaking back by the sphincter-action of this organ.

As a safe-guard, a second ligature was added, just posterior to the gizzard. A lid was usually sealed on to the watch-glass with petroleum jelly, and the preparation kept at room temperature or at 28° C. for 10–24 hr. A little sucrose was added to

the Ringer solution in the first trials, but was later omitted because, far from serving as a food for the tissues of the mid-gut, it enhanced bacterial growth. Overgrowth by bacteria was troublesome in many of the preparations, especially those kept at 28° C., and was partly counteracted in different preparations by adding thymol, merthiolate, penicillin (8000, 1000 or 500 units/c.c.) or streptomycin (1000 units/c.c.) + penicillin (1000 units/c.c.). None of these substances was found to be wholly satisfactory.

A total of fifteen trials was made. Eight of the trials were successful in that the cysts were found to be in some stage of hatching when the mid-gut contents were finally examined, and in one of them, *Lophomonas striata* had excysted. Seven of the successful preparations were among those kept at room temperature, but excystation was completed in only one. It was incomplete in the successful preparation at 28° C. This can probably be attributed to the bacterial overgrowth attacking the mid-gut before the cysts were sufficiently stimulated.

C. *Simple preparations with the mid-gut caeca.* The mid-gut caeca of one adult or large nymphal *Blaberus*, or of several *Periplaneta*, were dissected out and macerated in a little water. This constituted an extract of the enzymes of the caeca, and was used fresh, or after 1 or 2 days storage under refrigeration, together with M/15 pH 6·2 phosphate buffer. Cysts were picked out of a smear of faeces and saline, added to a little of the extract and a few drops of buffer on a slide; the preparation was sealed under a coverslip and kept at 28° C. for up to 12 hr.

One of the first trials was very successful: thirty out of sixty cysts were in various stages of hatching: this preparation had been made up with 20,000 units of penicillin. Eight attempts to repeat this success, sometimes varying or omitting the penicillin, were unsuccessful. An unusual feature of the cysts in this preparation was that they were the first to be produced after the host had moulted. This was probably irrelevant, because the same was true of the cysts used in one of the eight unsuccessful trials.

D. *Preparations with the mid-gut contents.* Similar preparations to those in C were made, but using the contents of the mid-gut, instead of the caeca, from several *Blaberus* or *Periplaneta*. Six trials were made at 28° C. and four at 25° C.; with and without buffer, and with and without penicillin. None was successful.

E. *Preparations with the fore-gut contents and suitable buffer.* In this series, the triggering of the process was assumed to occur in the fore-gut, with latent hatching in the mid-gut. Several cockroaches were fed infected faeces and, at various times afterwards, their fore-gut contents were dissected out on to a slide, diluted with saline or M/15, pH 5·0 acetate buffer, the coverslip sealed down and the preparation kept at 28° C. Other preparations, also kept at 28° C., were made with cysts picked out of faeces and put in the contents of the fore-guts of several cockroaches mixed with pH 5·0 buffer, or into buffer alone. No cysts hatched in any preparation.

F. *Preparations with buffers.* In this series, the pH of some part of the cockroach's gut was assumed to be the stimulus. Several preparations were made of fresh faeces diluted with M/15 phosphate buffer at pH 6·2 and 6·5 (approximately that of the mid-gut contents) and sealed under a coverslip. They were kept at 28° C. for up to 10 hr. but no cyst showed any sign of hatching.

Similar buffer solutions of pH 5·9–8·1 were tried under aerobic and anaerobic conditions at 25° and 28° C., but no *Nyctotherus* excysted. However, after 2 or more days, some *Monocercomonoides* did appear, and survived for several days, in the preparations on the alkaline side of, and at, neutral. This flagellate also appeared in control preparations, made up with 0·5 % NaCl instead of buffer.

A small number of *Nyctotherus* cysts did hatch in buffer solutions at pH 7·2 and 6·2, which were fortified with a little horse serum and kept under anaerobic conditions at 28° C. But they took 2 days to do so.

G. *Preparations with cysts recovered from the fore-gut.* Cysts were fed to a cockroach, recovered from its fore-gut contents, and treated with the extract of the mid-gut caeca in order to see if such a pre-treatment enhances the chances of successful excystation. Cockroaches were killed 2–14 hr. after being fed faeces, and the whole fore-gut contents, or just the cysts picked out of a smear of the fore-gut contents, were mixed with macerated caeca moistened with M/15 pH 6·2 phosphate buffer, sealed under a coverslip and the preparation kept at 28° C. Thirteen trials were made but only two were successful. Both of these preparations were made with cysts recovered 11 hr. after being fed; but other trials of cysts recovered after the same interval did not succeed. Some of the eleven unsuccessful trials were variations of the above method: the caeca preparation was diluted or omitted; the foregut contents and cysts were stored aerobically overnight, or were triturated in powdered glass, to simulate any grinding action that the gizzard might have, before being treated with the caeca and buffer.

Even in the successful preparations, excystation was not completed. The ciliates were fully formed and swimming within the cysts after 9 and 11 hr. respectively, but, although the preparations were maintained at 28° C. for the next 12 hr., the trophozoites never broke out. This suggests that the enzyme extracts were not active enough to weaken the knob effectively, although it was effective in stimulating excystation.

Five other trials were also unsuccessful. In these, the fore-gut contents from several cockroaches were dissected out on to a slide and cysts mixed with them, or the cysts were simply put in pH 5·0 acetate buffer, and left for variable times at 25° or 28° C. The cysts were then picked out and made up with mid-gut caeca and buffer, and kept at 28° C.

H. *Pretreatment with pure enzymes.* The agent responsible for the occasional success in the above experiments may have been one or other of the digestive enzymes in the gut of the cockroach, or the products of their activity. Swingle (1925) found that *Blatta orientalis* secretes amylase, invertase, maltase, lipase and protease. Wigglesworth (1928) confirmed this with *Blatta* and *Periplaneta americana* and found that there is also lactase and that the proteolytic enzymes are tryptase and peptidase.

Brief pre-treatment and prolonged treatment with salivary amylase and with a 2 % suspension of lipase were tried without success. Pepsin and papain seemed to be lethal in some cases and without any obvious effect in others. However, brief pre-treatment in a 0·2 % suspension of pancreatic trypsin produced a series of small but consistent successes.

The trypsin was mixed with an equal volume of M/15 phosphate buffer of pH 8·1 and several drops used to dilute a small quantity of infected faeces. The mixing was done on a cavity slide, the coverslip sealed down, and the preparation put in a 28° or a 37° C. incubator for times ranging from ¼ to 5 hr. At the end of the treatment, the coverslip was lifted and the cysts picked out and put in a drop of 0·5 % NaCl on a slide, the coverslip sealed and this preparation put in the 28° C. incubator.

The reaction of the cysts from different hosts was found to vary, even cysts from cockroaches of the same species and same stock. Some were killed very easily, others did not respond. However, in several preparations of cysts which had been treated with the enzyme for as little as 20 min. or as long as 5 hr., up to 5 % hatched, 5–9 hr. after being put in saline. In one preparation, treated for 1 hr. at 28° C., a single *Lophomonas striata* excysted within 16 hr. but no *Nyctotherus*.

(2) *Repetition and definition of the work of Lucas*

Lucas (1928) found that in *Nyctotherus ovalis*, the whole process of excystation is induced in 0·5 % NaCl solution by maintaining a temperature of 28–30° C. Her method was to mix fresh faeces and saline on a slide and seal down the coverslip. This preparation was observed on the stage of a microscope and maintained at the desired temperature by placing a carbon filament lamp a little distance away from the stage. She found that the most suitable temperature was 28° C. but a few cysts hatched at 30° C. They degenerated at 37°, did not respond at 25°, and a few started but did not complete the process at 26° C.

She remarked that 'This was a somewhat unexpected result. The cyst would not be subjected to such an influence on entering the host, unless it be that the digestive processes occurring in the stomach, result in a considerable evolution of heat'.

She does not state how long the cysts took to hatch *in vitro*. I presumed that she found no deviation from the figures she gave for *in vivo* excystation, namely 6–8 hr. At first I repeated her methods as exactly as possible, even to the extent of using a carbon filament lamp as source of heat. When this failed, variations were introduced, until finally I had some success.

Singly and sometimes in simple combinations, the following variations were tested, each experiment being repeated at least once and run for at least 8 hr., sometimes for as long as 24 hr.

(1) Preparations kept in incubators instead of under the lamp.

(2) Slides which were acid or detergent-cleaned.

(3) Infected faeces from *Periplaneta* (two stocks), *Blatta* (two stocks) *Blattella* and *Blaberus*.

(4) Temperatures of 25°, 26°, 28°, 29°, 30°, 32°, 37° C.

(5) Infected faeces from cockroaches which were maintained at room temperature, 25° and 30° C.

(6) Infected faeces stored at 4° and −18° C. for up to 14 and 3 days respectively.

(7) Infected faeces freshly squeezed through the anus of a cockroach and faeces up to a few days old.

(8) Hyper- and hypotonic salines; water.

(9) Brief pre-treatment in 1 % sulphuric acid or 1 % acetic acid.

(10) Aerobic and anaerobic conditions.

(11) Cysts washed clean of faecal matter.

(12) Infected faeces treated with mercuric chloride.

(13) Added penicillin.

(14) Added aerobic bacteria isolated from the fore-gut of a cockroach.

(15) Added bacteria from current and extinct cultures of *Nyctotherus* in horse serum and 0·5 % NaCl, 1:19.

(16) Added bacteria: one strain of *Proteus morganii*, two of *Aerobacter aerogenes*, three of *Escherichia coli*.

(17) Added solutions of glucose, sucrose, glycerol and peptone.

(18) Added yeast extract.

(19) Added infusions of Timothy grass hay which had been boiled or not, were fresh or up to 3 days old, had been Seitz filtered or not.

(20) Added 1 % solutions of the sodium salts of L-malic, citric, acetic, fumaric, oxalic, lactic, formic, stearic, or tartaric acid.

(21) Cysts lightly triturated in powdered glass.

The vast majority of these various preparations were quite unsuccessful. The use of the carbon filament lamp as compared to a suitable incubator seemed to make no difference to the results, but the former was used many times because it allows observation with least disturbance of the preparation during the course of the experiment.

It was found that a temperature of 37° C. for 10 hr. caused only an apparent degeneration: cysts so treated were fed to a cockroach and some at least excysted in the gut.

Eventually it was discovered that some of the cysts in some of the preparations would hatch at 28° C., if given sufficient time. The form of the preparation was merely the simple one used by Lucas; the period of exposure to a temperature of 28° C., 2 days. In this time, up to 50 % of the cysts might hatch, and the proportion rose to 90 % in 1 day more. Using the faeces produced by one infected *Periplaneta*, I measured the time taken for 75 % or more of the cysts to hatch at temperatures ranging from 25 to 37° C. The results are presented in Fig. 2. Although in every case, at least 2 days were needed for 75 % of the cysts to hatch, a smaller proportion excysted at 28°, 32° and 37° C. within 1 day, but at 25° C., 3 days passed before any appeared.

The shape of the curve in Fig. 2 suggests that the optimum temperature for this type of preparation is about 32° C. At temperatures below and above this, a longer time was taken; and at 37° C., there was a tendency for the cysts to degenerate after a few days instead of either hatching or remaining inert. Other experiments with cysts and faecal matter from nine other cockroaches strongly confirmed this. The nine hosts comprised three *Periplaneta*, four *Blatta*, and two *Blaberus*, but none produced cysts which hatched in such large numbers so regularly as the first *Periplaneta*. Indeed, the full 90 % of cysts in the faeces from some of these nine hatched only at 30 and 32°, in others only at 32° C.; and lesser

proportions, or none, at other temperatures. This was true even of two *Periplaneta* from the same stock as the first.

Monocercomonoides also appeared in the faecal preparations of the first *Periplaneta*. There was a steady increase in the numbers of this flagellate in each preparation on successive days, which suggests that once excystation has occurred, growth and division follow. At the different temperatures, *Monocercomonoides* first appeared at about the time when 75% or more of the *Nyctotherus* cysts had hatched: that is, if plotted on Fig. 2, the two curves would be very much alike. The exception was at 32° C.: *Monocercomonoides* appeared after 1 day, whereas it took 2 days for 75% of the *Nyctotherus* cysts to hatch.

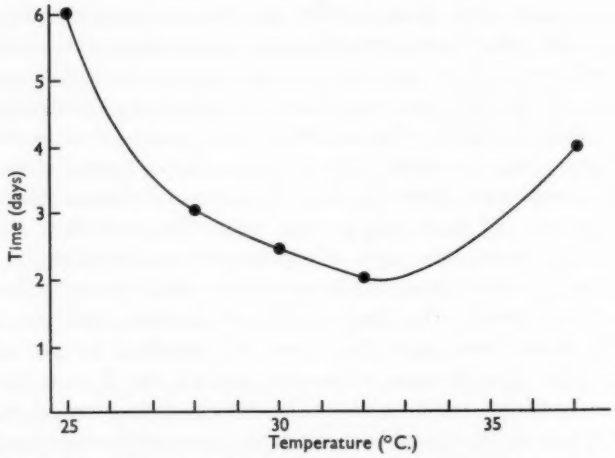

Fig. 2. *In vitro* excystation of *Nyctotherus ovalis*. The time taken for 75% or more of the cysts from one cockroach to hatch at temperatures between 25° and 37° C.

Endamoeba blattae and *Lophomonas striata* appeared to hatch only at 28° C., and then only in very small numbers after 3 and 1 days respectively. They appeared in the faeces only from the first *Periplaneta*.

Four types of experiments were undertaken to find out whether this type of *in vitro* excystation was a property of the faecal matter or an intrinsic property of the cysts. Faeces from the first *Periplaneta* were divided into two equal portions. One was made up as usual, but the other was made up with saline which had been boiled for a few minutes to drive off dissolved oxygen. At 28° C., the cysts hatched in both simultaneously, but thereafter a large proportion excysted in the preparation of boiled saline. This experiment was repeated, using the faeces from another cockroach. In the earlier experiments, cysts from this cockroach had been found to respond very poorly at any temperature; and in this experiment none hatched in either preparation.

In the second experiment, faeces from the first *Periplaneta* were diluted with 0·5% NaCl as usual, and some of the cysts picked out with a braking-pipette. Half were put in a drop of saline on a slide, and half in another drop containing 5000

units of penicillin. Coverslips were sealed and all three preparations put in the 32° C. incubator. There was a normal hatch in the first, control, preparation; later a few cysts hatched in the second preparation (of much diluted faeces), but none hatched in the preparation of penicillin. In a repetition of this experiment, the only cysts to hatch were in the control preparation.

Penicillin and streptomycin were used together and separately as simple additives to the ordinary faeces + saline preparations. 5000 units of each had the effect of delaying but not preventing the eventual hatching of the cysts. Used separately, they appeared to have no effect.

In the fourth experiment, faeces from two cockroaches were used simultaneously. The cysts in one were large and known to hatch easily (from the first *Periplaneta*); the cysts in the other were much smaller and rarely responded (from *Blaberus*). Both lots of faeces were diluted with saline and some of the cysts from each were picked out and put in the other. Both preparations were put in the 28° C. incubator. None of the *Blaberus* cysts hatched in either preparation. The *Periplaneta* cysts hatched readily when amongst their native faecal matter, but only one out of about a dozen hatched in the *Blaberus* faecal matter. This experiment was repeated, using faeces from the same *Blaberus* and from a *Blatta*. Only the *Blatta* cysts hatched and these only in their native faecal matter.

The results from these four types of experiment suggest that the property of *in vitro* excystation under these conditions resides not in the cysts themselves but in the flora of the faeces. The first experiment suggests that the bacteria are anaerobic; the second and third that they are inhibited by penicillin and by streptomycin if the concentration of bacteria is small, but if large, the antibiotics individually are without effect—presumably losing their potency more rapidly. Reduction of the concentration of bacteria in the preparations is in itself inhibiting.

The fourth experiment was intended to show that the factor responsible for excystation was produced by bacteria present in the faeces of the first *Periplaneta*, and to a lesser extent in the *Blatta*, but absent from the *Blaberus*. This interpretation of the results can only be accepted if it is assumed that the cysts from the *Blaberus* were more-or-less insensitive to the factor.

(*c*) *Discussion*

Much of the early work on *in vitro* excystation of parasitic Protozoa was done without taking precautions against contamination of the cysts by bacteria, or the cysts were frankly treated with faecal bacteria. Yorke & Adams (1926) demonstrated that *Entamoeba histolytica* will excyst in the media maintained at 37° C., showing that it is unnecessary to treat them with 'liquor pepticus' or 'liquor pancreaticus'. Their work has since been confirmed many times. The same conditions of moisture and a temperature of 37° C. were found to stimulate excystation in *Councilmania lafleuri* by Allen (1926), in *Iodamoeba williamsi* by Hegner (1927*a*) and Smith (1927); and in *Entamoeba coli* and *Endolimax nana* by Hegner (1927*b*), but he found that *Chilomastix mesnili* and *Giardia lamblia* did not respond so well.

The micro-organism which could stimulate *Schizopyrenus russelli* to excyst was found by Singh, Mathew & Sreenivasaya (1956) to be *Aerobacter* sp., strain 1912,

Singh (1941). The factor was thermolabile, could diffuse through a collodian membrane, and could not be replaced by reducing substances or agar adjusted to different levels of pH.

Working on an analogous problem, Poynter (1956) found that four different coliform organisms (*Escherichia*) from horse faeces could stimulate the second ecdysis of parasitic nematode larvae from the horse.

Entamoeba histolytica excysted under reduced oxygen tension in sterile media in the experiments of Snyder & Meleney (1941) and Chang (1946), the latter finding that the oxidation-reduction potential of the medium must be −115 mV. or less. The former found, however, that the presence of living bacteria increased the percentage of cysts which hatched, but even so this was never higher than 10%. Rees (1942) and Dobell (1952) obtained excystation with the same protozoan in media seeded with selected single species of bacteria. Rees, Reardon, Jones & Griffen (1947) obtained variable results with both *E. histolytica* and *E. coli* in a variety of sterile organic media. Addition of organism *t* to the media did not improve the results, nor did artificially lowering the oxygen tension. In further tests on *E. histolytica*, Rees, Reardon & Bartgis (1950) found that a small percentage of cysts hatched in inorganic media plus cysteine or glutathione; addition of amino acids made little difference; for a high percentage of excystation it was necessary to add a mixture of other organic compounds: B vitamins, vitamin C, cocarboxylase, purines, pyrimidines, folic acid, glucosamine and cholesterol.

In parallel with this work on parasitic organisms, numerous studies have been made on the cysts of free-living Protozoa. Excystation has been attributed to a variety of factors by authors working on different or even the same species. They were listed by van Wagtendonk (1955) as: (1) osmotic phenomena, (2) sufficiently high food and oxygen concentration, (3) enzymic dissolution of the membrane of the endocyst, and (4) the presence of organic end-products of protein decomposition. Many of these reports are based on poorly controlled experiments. However, all the factors were tried on *Nyctotherus* but gave negative results.

Haagen-Smit & Thimann (1938) studied the factors stimulating excystation in *Colpoda cucullus*. This ciliate will excyst in a variety of complex media, including hay-infusion. Haagen-Smit & Thimann showed that the activity of the ether-soluble fraction of the infusion was mostly due to the salts of L-malic, citric, acetic, fumaric, oxalic and lactic acids; that of the ether-insoluble, to a mixture of sugars. The activity of the two fractions combined was greater than either separately. In similar studies on the related *C. duodenaria*, Strickland & Haagen-Smit (1947) reported that potassium ion was the essential agent, and later (1948) showed that a mixture of 0·0001 M potassium phosphate solution and 0·3 methyl alcohol gave excellent results.

The physiological mechanism of excystation in any protozoan is still obscure. Van Wagtendonk (1955) summarized the little work that had been done and the theories that had been put forward. Briefly, it is supposed that the excystment medium supplies intermediates in enzymic pathways and cycles which became impaired at the time of encystation. The enzymes present in the encysted organism are triggered into activity and it is thus restored to the physiological status of the

free-swimming cell. The process then leads to redifferentiation of the protoplasmic structures, internal reorganization, and processes for the escape from the cyst membrane.

The work on the parasitic Protozoa can be fitted into this framework, but these organisms appear to be unable to live, in the excysted state, in an aerobic environment; consequently a reduced oxygen tension is an additional requirement for excystation. Indeed, it is this condition which makes it less likely that a parasite will excyst *in vitro* in the faeces of its former host and more likely that it will excyst successfully in its specialized environment of the gut of a new host.

A curious phenomenon which may be an integral part of excystation is the flowing to and fro of the cytoplasm which occurs at an early stage in the process. It was noticed, for instance, by Strickland & Haagen-Smit (1947) in *Colpoda duodenaria*, by Hegner (1927*b*) in *Entamoeba coli* and *Chilomastix mesnili*, by Dobell (1928) in *E. histolytica*, and (1938*a*), in *E. coli*. This flowing seems to occur immediately before the visible redifferentiation of cytoplasmic structures and suggests a physical re-alignment of their components. In the first phases, the movements probably hasten the last stages of the absorption of excystment factor and of water by the whole body of cytoplasm.

In *Nyctotherus*, in addition, the movement accomplishes a total re-orientation of the body within the cyst. This is all the more remarkable because it does not occur, according to Kidder (1937), in *N. uichanoi* of the wood-feeding roaches *Panesthia javanica* and *P. spadica*; and it must involve the establishment of a new polarity. This polarity was demonstrated in the trophozoites of *Nyctotherus hylae* by Rosenberg (1937). Addition of potassium cyanide to the medium resulted in first a breakdown of the co-ordination of the cilia at the apical end and then this spread progressively to the less sensitive posterior parts. This type of polarity has been demonstrated in many ciliates, notably by Child (1914) in *Paramecium*, *Stentor*, *Stylonchia*, *Vorticella* and *Carchesium*. This type of polarity seems to be distinct from that observed by Becker (1928) in *Mastigina hylae*: here the quality seems to reside only in a small cap at the tip of the advancing pseudopod, and the quality seems to consist only of an ability to induce a fountain streaming in the endoplasm behind the cap.

Fauré-Fremiet (1948) remarked that the notion of a 'morphogenetic field' is intuitive but the existence and activity of the infraciliature in ciliates are objective facts. His experimenal analyses of binary fission, or regeneration, and of the stability presented by double or multiple systems point to a relationship between the essential processes of morphogenesis in ciliates and their infraciliar mechanisms. *Nyctotherus* seems to be dominated by a single gradient of determinant, one of the main components of which is cyanide-sensitive and located in the cortex. However, no one has made satisfactory stained preparations of an encysted *Nyctotherus* and demonstrated whether or not the infraciliature is still present in a partly de-differentiated but organized state in the cortex. Certainly there is no sign of a gullet nor even of cilia. But the phenomenon of re-orientation does suggest that a ready-made organization is being pulled or pushed into a new position. The endoplasm flows round spasmodically but vigorously, and gives the impression of

moving the cortex by friction or a shearing-force. It must be admitted, however, that it is difficult to see whether the cortex is a real entity, that is, a permanent 'skin', and whether it actually moves; or whether it is merely part of the cytoplasm as a whole, as in a mobile amoeba. If the latter, the process of re-orientation must involve a migration of the infraciliature as elements through the body of the cytoplasm to their new positions, or a redifferentiation of the elements *de novo*.

The excystment factor itself seemed to be the trypsin produced by the cockroach, or the products of its activity. The number of cysts which hatched after treatment with pancreatic trypsin was disappointingly small. This may have been because the pH of the buffer used was optimum for this enzyme but, as Wigglesworth (1928) pointed out, the trypsin of the cockroach is active over a more acid range than pancreatic trypsin and it is the pH of the cockroach's mid-gut to which the cysts will be conditioned. The trypsin seemed to have the additional function of weakening the knob on the cyst so that the ciliate could push it off when it was ready to emerge.

The excystment factor was also produced by some anaerobic bacteria present in the faeces of some, but not all, specimens of cockroach. The simpler hypothesis is that there is only one factor and that the bacteria therefore produce a trypsin. Stephenson (1949) lists three families in which excretion of proteolytic exoenzymes into the medium most frequently occur: the Pseudomonadaceae, the Bacillaceae and some tribes of the Enterobacteriaceae, in particular the Proteae and the Clostridia. The first is composed mostly of aerobic bacteria, and the second of both aerobic and anaerobic bacteria; most *Proteus* are facultative aerobic organisms, and Clostridium spp. are anerobic or microaerophilic. Breed, Murray & Hitchens (1948) listed a number of bacteria which have been found by various workers in the gut of cockroaches, but these organisms were of uncertain identity and of unknown metabolic characters. Hatcher (1939) made tentative identifications of three types of bacteria found in the intestines of some *Periplaneta americana* but none fits the requirements. The fourteen bacteria found in the intestines of *Blattella germanica* by Jansen & Wedberg (1952) were all aerobic organisms. The identity of the responsible bacterium is therefore yet to be found.

This bacterium cannot be responsible for excystation *in vivo* because it has been shown to be present in only very small numbers in the faeces of some cockroaches, or wholly absent. This is not surprising, in spite of the exchange of flora which must occur in any population of cockroaches, in view of Jansen & Wedberg's statement that '...no single species was found to be common to all the roaches studied...'. A second reason for believing that it is the cockroach's enzyme and not the bacterium's which is responsible for *in vivo* excystation is that the temperature at which it can occur is much below the optimum for the bacterium. I assume that Lucas (1928) was dealing with a different strain of the same bacterium when she obtained *in vivo* excystation by incubating the faeces in 0·5% NaCl solution under anaerobic conditions. She found the optimum temperature to be 28° C., some four degrees lower than my data suggest; and she implied that excystation occurred in a much shorter time than the two days I found necessary.

It is problematical whether the flora of the cockroach's gut play even a minor part in stimulating excystation *in vivo*. Wollman (1926) reared *Blattella germanica* under sterile conditions but he did no work on the gut parasites. Wigglesworth (1927) suggested that the yeasts in the fore-gut were responsible for the acid conditions there (pH 4·6–6·0), but my evidence shows that passage through the foregut is irrelevant to excystation. The bacteria of the mid-gut may supply some factor or factors which enhance the response of the cysts to the host's trypsin. This might explain the failure of *Nyctotherus ovalis* to excyst in most of the arthropods and the frogs. Many of these are known to secrete a trypsin; but the pH of the different parts of their intestines may differ in some critical way from the cockroach, and this difference could inhibit the response of the cysts directly, or indirectly by way of inhibiting the growth of the flora of the cockroach's faeces fed along with the cysts; or perhaps more likely, since there was in any case probably insufficient time for the cockroach's bacteria to establish themselves, there were simply no suitable indigenous bacteria.

Two results which must be interpreted are the failure of *N. ovalis* to excyst in the gut of *Julus* and its mild success in the locust. Pai & Wang (1948) identified *N. ovalis* in a millipede they called '*Julus* sp.' of China, that is, a close relative of the *Julus terrestis* in which I found cysts of this ciliate from cockroaches failed to excyst. Either Pai & Wang were mistaken in their identification of the *Nyctotherus* and ought to have erected a new species; or, as seems more likely on studying their description of the *Nyctotherus*, *N. ovalis* is restricted to their *Julus* and the species of cockroach with which I have worked, and a different species occurs in *J. terrestis*.

The ability of a small proportion of the cysts to hatch after passage through the gut of a locust allows of two explanations. Two facts must be explained: only a very small proportion of the small-type cysts from *Blaberus* hatched, but up to 15% of the small-type cysts from *Periplaneta* and of the large-type cysts from *Periplaneta* and from *Blatta* hatched under the same circumstances. Secondly, some of the cysts degenerated after passage through the gut, instead of either hatching (or starting to) or remaining unaffected. If excystation is determined wholly by the trypsin, subject to a suitable pH, then the cysts from *Blaberus* must have been less sensitive than the others and the degeneration of the cysts was due to overstrong enzyme, an unsuitable pH, or some other simple cause.

On the other hand, it is striking that the cysts which were least able to hatch after passage through the locust are the same which are least able to hatch *in vitro* when incubated under anaerobic conditions with their native faecal matter. This suggests that the bacterium in the cockroach's faeces which was responsible for *in vitro* excystation had just time enough to establish itself and stimulate excystation in some of the cysts; and the degeneration amongst the cysts can again be explained by slightly unfavourable conditions. But two facts are against this interpretation: (1) faeces from *Blaberus* were fed simultaneously with faeces from the other cockroaches and were therefore subjected to the same conditions; (2) prolongation of the time spent by the cysts in the locust's intestines by ligating the posterior part of the locust's abdomen did not increase the proportion which

hatched but only increased the proportion degenerated. These two considerations suggest that there is a genuine difference in the sensitivity of the cysts from *Blaberus* compared to that of the cysts from other cockroaches; and that excystation in the locust, as in the cockroach, is a result of stimulation by the trypsin of the host, and the bacterium responsible for *in vitro* excystation plays no part. However, it is still possible that the flora of the cockroach supply some factor which enhances the response of the cysts.

The nature of this lesser sensitivity of cysts from *Blaberus* is unknown: it might reside in a less permeable knob, or the encysted organism may be physiologically different in that it is more inert. No suggestion of this lesser sensitivity was noticed during the work reported in an earlier section of this paper: cysts from *Blaberus* hatched in all four species of cockroaches quite as readily as did cysts from the other cockroaches. It is probable, therefore, that this difference is too slight to prejudice the chances of cysts from *Blaberus* against establishing infections in cockroaches.

Infections with *Nyctotherus* seem to be initiated by accident: there is no instinctive drive on the part of the cockroach to eat faeces. The form adopted by the ciliates in the new host seems to be dependent on the presence or absence of one or more types of bacteria which are typically found in *Blatta* but are absent from *Blattella*: the former species of cockroach harbours *Nyctotherus* which is larger, and contains large storage granules in greater or lesser numbers. In *Blattella*, the flora seems to be deficient in this bacterium which induces the large storage granules, and the *Nyctotherus* trophozoites and cysts are in consequence considerably smaller; the apical end of the trophozoite is more pointed and the cysts relatively more slender. This bacterium is not inevitably present in *Blatta*: cysts from one which were as small and slender as any from *Blattella* are described in the second paper of this series. These considerations show that the different types of *Nyctotherus* found in the different species of cockroach are merely phenotypic variations of a basic type found in *Blattella*. Many species of *Nyctotherus*, often differing from each other in only minor respects, have been described as occurring in various frogs: it is possible that these too are merely phenotypic variations of relatively few basic types.

III. SUMMARY

The only mode of transmission of *Nyctotherus ovalis* from one cockroach to another was shown experimentally to be by way of ingestion of the encysted stage of the ciliate. The cysts were remarkably resistant, being able to remain viable for 21 weeks at $-18°$ C. if kept in dry faeces, or for 20 weeks in wet faeces at 4°–25° C. Their life was much shorter if kept at 37°, 25°, or 4° C. under dry conditions, or at $-18°$ C. under wet. The cysts could withstand freeze-drying, or storage in pure oxygen, carbon dioxide or nitrogen, at least for short periods.

Nyctotherus ovalis could excyst in any of the four species of cockroach which were tested (*Periplaneta americana*, *Blatta orientalis*, *Blattella germanica*, *Blaberus giganteus*), irrespective of the species of origin. The ciliate could establish an infection in a very young host; in a host which had ingested infected faeces once

only; in hosts which were in a very dry or a very humid environment; in a host maintained at a temperature far below that required for *in vitro* excystation, subject only to the temperature being high enough for it to be possible for the cockroach to digest its meal; but *Nyctotherus* could not establish an infection in cockroaches which were about to moult, because there was no movement nor digestion of food in the gut. There was no evidence of an instinctive drive on the part of the cockroach to eat infected faeces. There was some evidence that the *Blatta* and *Blattella* types of trophozoite and cyst were associated with the presence or absence respectively of a type of flora typically associated with the hind-gut of *Blatta*.

The process of excystation took as little as 3 hr. for completion *in vivo*, but this time was increased if faecal material was ingested along with cysts. The process was most rapid in the smallest cockroach (*Blattella*) and slowest in the largest (*Blaberus*). The process, once started, could be completed *in vitro*, but at a considerably slower pace. Digestion of the knob on the cyst by the cockroach's enzymes seemed to be necessary if the ciliate was to emerge.

A small proportion of cysts hatched after passage through the gut of a locust, but no means were found of increasing this proportion, and an infection was never established. Excystation did not occur in any other arthropod, nor in the frog, which were tested.

Cysts recovered from the mid-gut of a cockroach completed the process of excystation *in vivo* more rapidly if they were put in a buffer medium of pH approximating that of the mid-gut than if they were put in saline. Passage of the cysts through the foregut of the cockroach had no function in stimulating excystation: the agent seemed to be the trypsin secreted by the mid-gut caeca, or the products of its activity. The many failures in the series of experiments which are reported here can probably be attributed to contaminating bacteria.

The complete process of excystation could occur *in vitro* if the cysts were incubated with faecal matter from the cockroach. The optimum temperature was 32° C., and 2 days were needed for a significant number to hatch. No means were found of shortening this time, nor any agents which could be substituted for the faecal matter. The stimulus seemed to be provided by particular anaerobic bacteria of the faeces which were present in some cockroaches, but absent in others.

Work by other authors on excystation in other Protozoa is reviewed. It was concluded that *in vivo* excystation of *Nyctotherus ovalis* occurs as a result of stimulation of the cyst by the digestive trypsin of the cockroach, and not by the products of the unidentified bacterium which is active *in vitro*. Cysts originating from *Blaberus* seemed to be less sensitive to this bacterial factor than some of those from *Periplaneta* or *Blatta*.

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The synonymy of *Ixodes festai* Rondelli 1926

By DON R. ARTHUR, Ph.D., D.Sc.
Department of Zoology, University of London King's College

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Ixodes festai was originally described from two Libyan and Tunisian female specimens from two different avian hosts by Dr M. Rondelli in 1926. Subsequently the female was re-described and the male and immature stages described from specimens collected in the Nefifik forest near Casablanca (Arthur, 1957*a*, 1958). Details of the life history were reported by Blanc & Bruneau (1958) and by Arthur (1958) from information furnished by Bruneau. More recently Morel (1959) has reported the occurrence of this species in France, but previously Gil Collado (1936, 1948) described it as a Spanish species under the name of *Ixodes ventalloi*.

Prior to having access to the material of *I. festai* from Morocco, I (Arthur, 1955, 1957*b*) reported the occurrence of an allegedly new species, viz. *I. thompsoni* from domestic cats on Lundy Island. On comparing the latter with *I. festai* I find they are the same species. Accordingly, *I. thompsoni* and *I. ventalloi* must be sunk in synonymy with *I. festai*. This means that the geographical range of this species now takes in Britain, France and Spain as well as North Africa, and the host range is extended to include the domestic cat—the only known host in Britain. Doubtless with further intensive collecting at Lundy Island the true wild host will be found.

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Certain aspects of the host-parasite relationship of *Nematospiroides dubius* (Baylis)

II. The effect of sex on experimental infections in the rat (an abnormal host)

By COLIN DOBSON*

Department of Zoology, The University, Sheffield* 10

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INTRODUCTION

It is now well known that the susceptibility of the male host to parasitic infection differs from that of a female host (Hunninen, 1935; Culbertson, 1941; Whitlock, 1937; Clapham, 1939; Campbell, 1939; Campbell & Melcher, 1940; Chandler, 1943; Addis, 1946; Todd & Hollingsworth, 1952; Sadun, 1948, 1951; Mathies, 1954, 1959; Haley, 1958; Dobson, 1961). From these various sources it is evident that the male host is more susceptible to infection than the female; oestrogen tending to decrease the susceptibility, while testosterone has little or no effect.

Since it was found possible, by the use of young animals, to establish infections of *N. dubius* in the rat, despite the contrary observations of Cross & Scott (1956) and Cross (1960), an investigation into the effects of sex in this abnormal host was undertaken in order that a comparison with the results previously obtained for the mouse (Dobson, 1961) could be made.

MATERIALS AND METHODS

All the animals used were from a small randomly mated colony of Wistar strain rats, free from helminthes. The first oestrus occurred between 45 and 50 days. The infective nematode larvae were cultured and counted; the hosts were infected by stomach tube, maintained, slaughtered and treated by the methods previously described (Dobson, 1961). The rats were 25, 38 and 100 days old when they were infected, each with one hundred infective larvae.

The rats were grouped into three units, bilateral gonadectomy being performed on two of them. This was done under general ether anaesthetic, the ovary being

* Present address: Department of Parasitology, University of Queensland, Brisbane, Australia.

approached via a dorsal incision and the testis through an incision in the ventral body wall just anterior to the penis.

One group of the gonadectomized rats had pellets of the homologous sex hormone implanted in them subcutaneously. These pellets were manufactured by the Organon Laboratories as testosterone and 1:9 oestradiol/cholesterol. The implants were weighed before implanting and after removal; the average absorption of testosterone appeared to be about 0·3 mg./day and of oestradiol 2–4 μg./day. These figures are in general agreement with the amounts calculated from the known rates of absorption in man in relation to the surface area of the implant and with the data given by Ebling (1957). The data of the manufacturers suggest that, on average, 0·2 mg. testosterone and 3 μg. oestradiol would be absorbed each day.

A statistical analysis of all the data was made using Student's 't' test. The calculated t values are given in the tables and their corresponding P values in the text.

RESULTS

I. *Immature rats*

(*a*) *Experimental infections in normal 25-day-old animals*

Infections lasting 5 days. More worms were recovered from the female than from the male rat ($P = < 0{\cdot}001$). There was, however, no significant difference between the male and female in the percentage lengths of the small intestine infected ($P = 0{\cdot}5$–$0{\cdot}4$) (see Table 1).

Infections lasting 10 days. There was no difference between the worm burdens of male and female rats ($P = 0{\cdot}9$–$0{\cdot}8$). The male harboured more worms ($P = < 0{\cdot}1$), while the female harboured fewer worms ($P = < 0{\cdot}05$) on the tenth day than were observed on the fifth day of the infection (see Table 1).

The development of the worm was, therefore, slower in the male than in the female rat, but, although the female rat was initially more favourable to the parasite, it showed a marked resistance to the infection by the tenth day which the male rat did not show (see Table 1).

By counting the numbers of cystic scars along the small intestine on the tenth day of the infection, an estimate of the numbers of larvae which initially penetrated the gut wall was obtained. More cystic scars were found in the male than in the female rat ($P = < 0{\cdot}001$). It is evident from this that the male rat is more susceptible to infection. Also, since only 25% of the larvae which infected the male rat formed cysts by the fifth day compared with 100% in the female rat, the development of the worm must be slower in the male than in the female rat (see Table 1).

(*b*) *The effects of gonadectomy on infections in the 25-day-old rat*

In order to determine some of the factors causing the differences between the susceptibilities of the male and female rats, infections were established in rats gonadectomized when they were 21 to 23 days old. The control animals for the following experiments were identical with those from the previous experiment; all

Table 1. *The worm burdens and the lengths of the small intestine infected in 25-day-old control and experimental rats, infected for 5 and 10 days, with 100 infective larvae of* Nematospiroides dubius

Table 1. *The worm burdens and the lengths of the small intestine infected in 25-day-old control and experimental rats, infected for 5 and 10 days, with 100 infective larvae of* Nematospiroides dubius

Sex of the host and duration of the infection in days		No. of rats used	No. of cysts or cystic scars	't' values	No. of adult worms	't' values	No. of male worms	No. of female worms	't' values. No. of male to no. of female worms	% length gut infected	't' values
Male*	5	8	11·7 ± 1·79	2·07	—		—	—	—	25·15 ± 5·4	1·21
Castrate male	5	8	19·13 ± 3·95	4·06	—		—	—	—	32·75 ± 6·4	1·77
Treated castrate male	5	7	21·86 ± 3·04	1·09	—		—	—	—	42·95 ± 10·13	1·29
Female	5	9	42·67 ± 8·06	4·05 1·5	—		—	—	—	29·99 + 10·22	0·54 0·17
Spayed female	5	7	27·43 ± 6·77	2·94 3·48	—		—	—	—	31·46 ± 4·31	1·60 0·47
Treated spayed female	5	8	48·76 ± 9·66	3·09 1·14	—		—	—	—	32·49 ± 7·71	0·32 0·07
Male	10	6	60·33 ± 4·26	1·71	27·2 ± 7·5	2·03	12·8 ± 2·64	14·4 ± 4·76	−0·41	9·36 to 62·37 ± 3·27 ± 2·58	— 3·23, 0·61
Castrate male	10	6	48·33 ± 8·21	3·09 0·57	36·5 ± 5·93	2·75 1·87	15·33 ± 2·08	21·17 ± 4·5	−1·36	2·58 to 60·65 ± 2·51 ± 5·13	2·49, 0·61 0·47, 0
Treated castrate male	10	6	52·0 ± 4·73	5·39 0·95	44·83 ± 3·91	0·22 2·10	17·16 ± 3·97	24·16 ± 2·7	−2·41	1·43 to 60·68 ± 0·64 ± 5·13	1·94, 3·82 1·03, 3·44
Female	10	8	43·14 ± 2·82	1·21 7·58	28·5 ± 4·43	9·41 6·03	14·0 ± 1·46	14·25 ± 3·95	−0·68	13·91 to 52·67 ± 4·02 ± 3·62	2·26, 5·19 3·46, 1·27
Spayed female	10	7	55·8 ± 2·27	2·33 39·29	47·0 ± 3·19	6·33 7·19	23·83 ± 1·68	23·16 ± 2·89	−0·26	0·0 to 45·43 ± 6·77	0·62, 0·88 2·54, 0·91
Treated spayed female	10	8	47·0 ± 2·28		24·25 ± 4·48		13·12 ± 1·10	13·68 ± 3·67	−0·16	12·25 to 48·87 ± 4·83 ± 5·62	

* Comparisons between the various types of mice after 5 and 10 days infection respectively: male, 2·11; castrate male, 3·93; treated castrate male, 9·34; female, 2·11; spayed female, 3·28; treated spayed female, 2·86.

the rats were treated at the same time and the results segregated for the convenience of presentation.

Infections lasting 5 days. More worms were encysted along a greater length of the small intestine in the castrate male than in the normal male rat ($P = < 0{\cdot}05$). The normal female rat harboured fewer worms along a similar length of the intestine than the spayed female rat ($P = 0{\cdot}01$–$0{\cdot}001$), while there was no difference between the worm burdens of the castrate male and spayed female rats ($P = 0{\cdot}2$–$0{\cdot}1$) (see Table 1).

The basic sex ratio of the parasites was predominantly female in both gonadectomized and normal hosts (67 % female parasites from the spayed female, 65 % from the castrate male, 62·9 % in the normal male and 61·8 % from the normal female rat).

Infections lasting 10 days. Although fewer cystic scars were found along the small intestine in the castrate male than in the normal male rat the difference was not significant ($P = 0{\cdot}2$–$0{\cdot}1$). Of the larvae which had penetrated the intestinal wall in the castrate male (i.e. the number of cystic scars), more had formed cysts by the fifth day than in the normal male rat (39·6 % compared with 19·7 %). The castrate male harboured more adult worms than the normal male rat, but although this difference suggested a marked increase it was not significant ($P = 0{\cdot}1$–$0{\cdot}05$) (see Table 1). The number of cystic scars along the small intestine of the spayed female rat was not significantly different from that found in the castrate male ($P = 0{\cdot}4$–$0{\cdot}3$), but it was greater than the numbers of scars found in the normal female rat ($P = < 0{\cdot}001$). On the other hand, fewer of these larvae formed observable cysts by the fifth day of the infection in the spayed female and castrate male than in the normal female ($P = < 0{\cdot}001$) (49·2 % in the spayed female compared with 98·9 % in the normal female and 39·58 % in the castrate male rat).

Similar numbers of adult worms were found in the spayed female and castrate male rat ($P = 0{\cdot}1$–$0{\cdot}05$) although fewer survived to the adult stage in the normal female than in the spayed female rats ($P = < 0{\cdot}001$) (88·26 % in the spayed female compared with 59·36 % in the normal female and 75·52 % in the castrate male rat) (see Table 1).

The ratio of male to female parasites was about unity in all groups of hosts after 10 days infection (52·9 % female worms in the normal male, 59·5 % in the castrate male; 50·0 % in the normal female and 49·3 % in the spayed female rats).

(*c*) *The effect of gonadectomy with replacement of the homologous sex hormone on experimental infections in the 25-day-old rat*

This experiment represents a study of the effects of replacement of the sex hormone in gonadectomized animals on experimental infections.

Testosterone did not decrease the worm burden of the castrate male rat to that found in the normal male rat ($P = 0{\cdot}3$–$0{\cdot}2$). A few more female than male worms were recovered from their cysts along the small intestine (53 % female worms).

Oestradiol increased the worm burdens of the spayed female rats to that found in the normal female hosts ($P = 0{\cdot}3$–$0{\cdot}2$). The treated spayed female rat harboured significantly more encysted larvae than the treated castrate male rats ($P = < 0{\cdot}01$).

This was a similar relationship to that shown by the normal hosts (see Table 1). Equal numbers of male and female encysted larvae were recovered from the treated spayed female rat.

Infections lasting 10 *days.* As estimated by the numbers of cystic scars found, testosterone did not increase the number of larvae which penetrated the intestinal mucosa in the castrate male up to the level found in the normal male rat (P = 0·7–0·5): neither did it affect the rate of development of the worm (only 42·1 % of the larvae which ultimately formed cysts had done so by the fifth day in the treated castrate male, compared with 60·3 % in the normal male) (cf. Table 1). However, more worms were recovered as adults from the treated castrate male rat than from either the castrate or normal male rats (P = 0·02–0·01 and 0·1–0·05). Thus testosterone increased both the numbers of larvae which penetrated the gut wall and of those which survived to the adult stage in the castrate male rat.

The ratio of male to female worms was about unity in the treated castrate male rat.

Oestradiol decreased the numbers of larvae which penetrated the intestinal wall in the spayed female rat to a number similar to that in the normal female rat (P = < 0·001). The numbers were also smaller than in the treated castrate male rat, but not significantly so (P = 0·3–0·2).

Oestradiol increased the rate of development of the parasite by the fifth day of the infection in the spayed female rat to a level similar to that shown by the normal female rat (P = 0·01–0·001) and consequently produced a higher developmental rate than was shown in the treated castrate male rat (P = 0·01–0·001), (100 % of those larvae which penetrated the gut wall in the treated spayed female rat had formed cysts by the fifth day compared with 42·1 % in the treated castrate male, 49·2 % in the spayed female and 98·9 % in the normal female rats).

Since significantly fewer adult worms were recovered from the treated spayed female than from the spayed female rat, oestradiol reduced the numbers of larvae surviving to the adult stage to a level similar to that found in the normal female rat (P = < 0·001). Fewer adult parasites were also found in the treated spayed female rat than in the treated castrate male rat (P = 0·001) (see Table 1).

Fewer of the larvae which had penetrated the gut wall survived as adults in the treated spayed female than in the spayed female rat, but there was no difference from those surviving in the normal female rat (45·3 % compared with 88·3 % in the spayed female, 86·2 % in the treated castrate male and 59·3 % in the normal female rats).

The sex ratio of the worms was about unity in both the treated castrate and treated spayed female rats.

(d) The effect of oestradiol on the resistance of the 38-day-old castrate male rat to experimental infection

Oestradiol was more effective in increasing the resistance of the rat to infection than testosterone. To show conclusively that oestradiol was the factor responsible for this increase, a study was made of the effect of this hormone on infections in the castrate male rat. Although these rats were slightly older (38 days old when

infected) than those previously used, they were castrated at the same age, viz. 21–23 days old.

Many more larvae penetrated the gut wall (measured by the cystic scars found) in the control male rat than in the treated castrate ($P = <0{\cdot}001$). Adult worms were more numerous in the control male rat (see Table 2).

Thus oestradiol increased the resistance of the castrate male rat above that shown by the control male rat. Also, the control male rats, although only 14 days older than the rats previously used in these experiments, showed a marked age resistance.

Table 2. *The worm burdens of control and treated castrate* 38*-day-old male rats infected for* 5 *and* 10 *days with* 100 *infective larvae of* Nematospiroides dubius

Sex of the host and duration of the infection in days	No. of rats used	No. of cysts or cystic scars	't' values	Adult worm burden	't' values	No. of male worms	No. of female worms	't' values. No. of male to no. of female worms
Male 5	7	15·57 ± 2·27	2·94	—		—	—	—
Treated castrate male 5	8	10·62 ± 1·53	9·46	—	5·76	—	—	—
Male 10	7	34·14 ± 1·14	8·22 5·1	5·43 ± 1·45	8·11 3·42	4·14 ± 1·02	1·29 ± 0·59	4·13
Treated castrate male 10	7	24·29 ± 2·26		2·57 ± 1·17		1·80 ± 1·43	1·14 ± 0·67	0 41

II. *Mature rats*

(*a*) *Experimental infections in normal,* 100*-day-old rats*

Since it has already been found possible to infect young rats with *N. dubius* and that rats become resistant with age, a study was made of the effects the sexual maturity of the rats had on the infection.

Infections lasting 5 *days.* More of the larvae encysted along the small intestine in the female than in the male rat ($P = <0{\cdot}001$) (see Table 3).

Infections lasting 10 *days.* More adult parasites were recovered from the male than from the female rat ($P = 0{\cdot}3$–$0{\cdot}2$), indeed the female rat was clear of any infection. The adult worm burdens of both male and female rats were smaller than the numbers of encysted larvae counted on the fifth day of the infection ($P = <0{\cdot}001$) (see Table 3).

Fewer larvae actually penetrated the gut wall in the female than in the male rat although the difference was not significant ($P = <0{\cdot}3$). The development of the worm was slower in the male than in the female rat ($P = <0{\cdot}001$), but the female rat had a greater resistance to the infection by the tenth day than the male since it harboured no adult worms. This relationship is similar to that found in the immature rats.

(*b*) *The effects of gonadectomy on experimental infections in the* 100*-day-old mature rat*

All the animals were gonadectomized when they were 21–23 days old. The control animals are identical with those used in the previous experiment and were infected at the same time as these animals.

Infections lasting 5 *days.* More worms formed cysts in the castrate male than in the normal male rat although the difference was not significant (P = 0·9–0·8). Fewer larvae formed cysts in the spayed female than in the normal female rat (P = < 0·001), but there was no difference between the numbers of worms from the gonadectomized animals, although more worms were recovered from the castrate male rat (P = < 0·05) (see Table 3).

Table 3. *The worm burden of male and female mature* 100*-day-old control and experimental rats infected for* 5 *and* 10 *days with* 100 *infective larvae of* Nematospiroides dubius

Sex of the host and duration of the infection in days	No. of rats used	No. of cysts or cystic scars	't' values	No. of adult worms	't' values
Male 5	5	22·6 ± 3·13		—	—
			1·64		
Castrate male 5	5	24·6 ± 3·36	1·20	—	—
			0·85		
Treated castrate male 5	5	22·0 ± 1·41	12·44	—	—
			2·54		
Female 5	5	27·0 ± 3·34	4·0	—	—
			3·38		
Spayed female 5	3	17·0 ± 1·54	2·97	—	—
			1·85		
Treated spayed female 5	2	18·0 ± 1·41		—	—
Male 10	6	53·0 ± 6·33	0·94	4·6 ± 4·57	1·01
Castrate male 10	8	47·0 ± 1·75	2·36	0	0·89
			3·79		1·97
Treated castrate male 10	8	63·85 ± 4·78	1·08	0·57 ± 0·29	1·01
			0·36		
Female 10	8	46·5 ± 2·82	9·81	0	
			0·29		
Spayed female 10	8	45·62 ± 4·20	3·08	0	
			5·79		
Treated spayed female 10	8	37·85 ± 3·95		0	

Infections lasting 10 *days.* There were fewer cystic scars along the intestine of the castrate male than in the normal male rat, but the difference was not significant (P = 0·4–0·3). More larvae did, however, form cysts in the castrate male rat after 5 days infection (P = 0·1–0·05) (52·3% compared with 42·6% in the normal male). While a few larvae survived to the adult stage in the normal male rat none did so in the castrate male (P = 0·4–0·3) (see Table 3).

Similar numbers of cystic scars were found in both the normal and spayed female rats ($P = 0{\cdot}8$–$0{\cdot}7$). However, fewer of the larvae which had penetrated the intestinal wall formed observable cysts in the spayed female than in the normal female rat by the fifth day ($P = < 0{\cdot}001$). There was no difference between the numbers of encysted larvae in the gonadectomized rats ($P = 0{\cdot}5$–$0{\cdot}4$); (37·3 % of the larvae which had penetrated the gut wall formed cysts by the fifth day in the spayed female and 53·3 % in the castrate male rat). There were no adult parasites recovered from either the spayed female or normal female rats.

(c) *The effect of gonadectomy with replacement of the homologous sex hormone on experimental infections in the* 100*-day-old mature rat*

Infections lasting 5 *days.* Testosterone did not alter the worm burdens of the castrate male rat ($P = 0{\cdot}9$–$0{\cdot}8$); neither did oestradiol in the spayed female ($P = 0{\cdot}2$–$0{\cdot}1$). The treated castrate male harboured significantly more worms than the treated spayed female rat ($P = < 0{\cdot}001$) (see Table 3).

Infections lasting 10 *days.* Fewer cystic scars were found in the normal male ($P = 0{\cdot}5$–$0{\cdot}02$) and in the castrate male ($P = < 0{\cdot}001$) than in the treated castrate male rats. Fewer of these larvae formed observable cysts by the fifth day of the infection in the treated castrate male rat than in either the normal or castrate male rats (34·5 % compared with 42·4 % in the normal male and 52·6 % in the castrate male rat).

Testosterone increased the survival of the worm to the adult stage ($P = 0{\cdot}1$–$0{\cdot}05$) and brought it to a level similar to that in the normal male rat ($P = 0{\cdot}3$–$0{\cdot}4$).

Oestradiol decreased the susceptibility of the spayed female rat to the infection ($P = < 0{\cdot}001$). Fewer cystic scars were also found than in either the treated castrate male ($P = < 0{\cdot}001$), or the normal female rat ($P = < 0{\cdot}001$) (see Table 3). The rate of development of the parasite in the spayed female rat, as well as the resistance of the host, was increased by oestradiol. This effect was greater than that in the treated castrate male, since the male did harbour a small number of adult worms.

DISCUSSION

Dobson (1961) reported that the mouse showed a marked sex resistance to infections with *Nematospiroides dubius*. Similar experiments using the rat as host have shown that the male was more susceptible to infection than the female, since initially more larvae penetrated the intestinal mucosa to form cysts in the male rat. Also, although the female rat harboured more observable cysts by the fifth day of infection, the male harboured a greater (but not significantly greater) number of adult parasites.

Twenty-five-day-old mice (Dobson, 1961) showed a greater sex difference in their resistance to infection than rats of the same age. This was probably due to the fact that the mice mature sooner than the rats. The 25-day-old mouse must, therefore, possess more circulating sex hormone than the rat of the same age, hence the great sex difference in their resistance to infection.

Cross (1960) stated that the natural resistance of the rat to infections with *N. dubius* appears to have several aspects. First, the connective tissue reaction which affects the developing worm in the intestinal wall; secondly, a lower percentage of worms develop in the rat than in the mouse; and third, a somewhat more extended prepatent period than in the mouse. However, at no time does he mention the ages of the rats he used and, since age is an extremely important factor when abnormal host-parasite relations are being studied, his results lose their validity. The present experiments have shown: first, that the young rat is not naturally resistant to infections of *N. dubius*; secondly, that the development of the parasite is not slowed until after it returns to the lumen of the gut: thirdly, that fewer larvae actually penetrated the intestinal wall initially in the rat than in the mouse.

The resistance of the rat thus depends, firstly upon preventing the larvae penetrating the intestinal wall, then secondly and mainly, upon the cellular reactions against the encysted larvae. The formation of a connective layer around the larvae is obviously a major resistance mechanism since a highly vascular area of opaque connective tissue was observed around the larvae in the rat, but not in the mouse. There must, nevertheless, be a certain critical thickness of connective tissue below which the worm can penetrate, since adult worms returned to the lumen of the intestine in these rats.

The results of Cross (1960) would be in agreement with this if they refer only to fully adult rats.

Male rats were shown to be more susceptible to infection than the females. When these hosts were gonadectomized the difference was removed and subsequent replacement of the homologous sex hormone restored, and indeed increased, the difference. This could be expected since the normal rats which showed the greatest susceptibility to the infection were very juvenile. Oestradiol increased the resistance of the spayed female, while, on the other hand, testosterone increased the susceptibility of the castrate male rat. Since it appeared that the difference in resistance to infection shown by the sexes depended mainly upon the presence of oestradiol, testosterone taking little or no part, confirmation was sought by infecting castrate male rats treated with oestradiol. This increased the resistance of the castrate male rat compared with that in the normal male.

These results are in general agreement with those of Campbell & Melcher (1940), von Haam & Rosenfeld (1942), Sadun (1948, 1951), Haley (1958), Mathies (1959) and Dobson (1961), who showed that oestrogen increased the resistance of the host to infection while testosterone had little or no effect. The cellular reaction and the subsequent encapsulation of the larvae in the intestinal tissues appear to be similar to the reactions described by other authors. Taliaferro & Sarles (1939) reported that in infections with *Nippostrongylus muris* of hyperimmunized rats, the larvae became encapsulated and precipitates were found in and around them, the immunity of the rats being primarily humoral with cellular co-operation. Larsh & Race (1954) came to a similar conclusion with reinfections of *Trichinella spiralis* in hyperimmunized mice. Similarly, Lindquist (1950) showed that the retention of the larvae of *N. muris* in the skin and lungs of an abnormal host, the hamster, was due to reactions which resembled those described by Taliaferro & Sarles (1939).

However, he was unable to demonstrate the precipitin reactions associated with the larvae which they had described.

When Cross (1960) demonstrated that the resistance of the rat to *N. dubius* infections could be overcome by the injection of cortisone, he concluded that the resistance reactions of the rat were primarily cellular. Similarly, Stoner & Godwin (1953) and Coker (1955) found that cortisone increased the susceptibility of mice to *Trichinella spiralis* infections, while it decreased the cellular responses of the intestinal tissues. Weinstein (1955) found that cortisone administered to rats immunized against *N. muris* caused a marked reduction in the cellular responses of the skin to infections. Roman (1956) demonstrated that adult mice, usually resistant to *Strongyloides ratti*, could become infected if they were treated with cortisone, while Olson (1957) showed that treatment of the white rat with cortisone, an abnormal host of *Litomosoides carinii*, resulted in a decrease in the numbers of this worm encapsulated in the pleural cavity.

Since differences in the connective tissue reactions due to the actions of the sex hormones can be initiated equally in the rat and mouse, it follows that infections in the weanling normal rat would not be different in the male and female since they have little circulating sex hormone, while the sex difference in the gonadectomized rats treated with the homologous sex hormone was due to the differential actions of these hormones on the formation of connective tissue. The fact that fewer larvae penetrated the intestinal wall in the rat than in the mouse could be explained either on the assumption that the penetrating enzymes of the larvae were at least partly specific for the tissues of the mouse, or else that the larvae were unable to penetrate the thicker tissues of the rat intestine (Lewert, 1958). Once the larvae had penetrated the intestinal wall they would have difficulty in returning to the lumen of the intestine for similar reasons.

SUMMARY

1. The male rat is more susceptible to infections of *Nematospiroides dubius* than the female. As the rat grows older the resistance of the female rat to infection increases at a greater rate than that of the male.

2. The course of the infection is modified by the sex of the host.

3. More larvae penetrated the intestinal mucosa to encyst in the male than in the female. More larvae, however, formed cysts in the female than in the male rat by the fifth day.

4. The male harboured more adult worms than the female rat, although this difference was not significant in the immature animals.

5. The sex resistance of the rat to *N. dubius* infections was removed by bilateral gonadectomy. Castration decreased the susceptibility of the male rat, while spaying increased it in the female compared with the susceptibility in the respective normal hosts.

6. Subsequent replacement of the homologous sex hormone in the gonadectomized rat restores the sex resistance, and may even increase it (particularly in the immature animals). Oestradiol increased the resistance of the spayed female rat, while testosterone increased the susceptibility of the castrate male rat to infection.

7. Oestradiol implanted in castrate male rats increased the resistance of these hosts to a greater level than was shown in the normal male rat.

8. The rat shows a marked age resistance over which the sex resistance is superimposed.

9. The relationship between the sex of the host and its resistance to infection is discussed.

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Certain aspects of the host–parasite relationship of *Nematospiroides dubius* (Baylis)

III. The effect of a milk diet on experimental infections in the adult male mouse

By COLIN DOBSON*

Department of Zoology, The University, Sheffield 10

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INTRODUCTION

Suckling 10-day-old mice infected with *Nematospiroides dubius* were found to yield lower worm burdens than the weanling 15-day-old mice fed on a solid diet (see Table 1). Woodland (1924), Shorb (1933), Hunninen (1935) and Larsh (1943) found that young mice were less susceptible to infections than older mice, Hunninen stating that the shortness of the intestine and the small size of the villi act as a mechanical disadvantage to the normal penetrating activities of the onchosphere of *Hymenolepis diminuta*.

Alternatively, the low susceptibility shown by the suckling mouse to *N. dubius* infections could be due to the milk diet, since Rohrbacher, Porter & Herlick (1958) found that calves fed solely on a milk diet harboured fewer *Haemonchus placei* than calves fed milk, hay and grain, and that fewer *Trichostrongylus axei* were harboured by suckling than by weanling rabbits. Similarly, Leese (1943) showed that cow's milk given to colts infected with strongyloids was highly beneficial to the animals, while Shorb & Spindler (1947) and Spindler, Zimmerman & Hill (1944) reported that when parasitized pigs were maintained on skimmed milk for 3–5 days most of the nematodes were expelled and the pigs made better weight gains. However, Porter (1935) found that greater numbers of *Nippostrongylus muris* developed in rats on whole milk than in rats on good stock rations, and Foster (1936) observed that dogs and cats fed on milk were more susceptible to hookworms than animals on normal diets.

Thus, since the evidence suggests that the lowered susceptibility to infection of the suckling mouse could be due to either of the two factors mentioned above, experiments were carried out to determine the effect of a milk diet on infections in adult male mice.

* Present address: Department of Parasitology, University of Queensland, Brisbane, Australia.

MATERIALS AND METHODS

All the mice used were from a Swiss strain colony, free from helminthes; only males were used to eliminate differences in the susceptibility of the host due to sex (Dobson, 1961). The infective nematode larvae were cultured and counted; the mice infected by stomach tube, maintained, slaughtered and treated using the methods previously described (Dobson, 1961). Mice aged 10 and 15 days, together with mature male mice, were infected with fifty infective larvae for 5 and 10 days.

Before the mature mice were fed on milk the bacterial flora associated with the rat cake diet was eliminated by feeding a mixture of 200 mg. Sulphamezathin and 6·25 mg. Pycimethumin in 100 g. of dried rat cake. This mixture was prepared following the method of Beverley & Anderson (1961).

Two hundred grams of rat cake were ground up in a mincing machine and the required concentrations of the drugs added and mixed thoroughly in the dry state with a pestle in a mortar. One hundred and twenty ml. of water were then added and the diet remixed. The consistency of the material was then like wet soil and was suitable for casting. The wet pellets formed by the casting machine were dried overnight at 60° C. Approximately 200 g. of the pellets was sufficient to feed six mice for 5 days.

The experimental mice were fed on the medicated rat cake for about 14 days before being fed on cow's milk for a further 7 days prior to being infected.

RESULTS AND DISCUSSION

Male mice harboured more worms when fed on cow's milk than when fed on a rat cake diet for infections of either 5 or 10 days ($P = < 0{\cdot}001$ and $= 0{\cdot}05$–$0{\cdot}02$). This increase in the susceptibility occurred without any alteration in the predominantly female sex ratio shown by the worms ($P = < 0{\cdot}001$ and $= 0{\cdot}1$–$0{\cdot}05$), or the ecology of the worm in the small intestine ($P = < 0{\cdot}9$ and $0{\cdot}4$–$0{\cdot}3$) (see Table 1).

Thus we see that mature male mice were more susceptible to infection when fed on cow's milk than when fed on rat cake, since significantly more worms were recovered from the mature male mouse on the milk diet. The small worm burdens of the suckling mice would not, therefore, appear to be due to the milk diet, but rather supports the hypothesis of Hunninen (1935) and Larsh (1943), that the shortness of the intestine and the small size of the villi act as a mechanical disadvantage to the normal penetrating activities of the larvae. Thus fewer larvae can survive the rapid passage through the intestine of the suckling mice; their milk diet, being liquid, may well accentuate the speed of the intestinal clearance.

SUMMARY

1. The suckling 10-day-old mouse harboured fewer worms than the weanling 15-day-old mouse after both 5 and 10 days' infection.

2. The adult male mouse, however, harboured significantly more worms when fed on milk than when fed on rat cake after both 5 and 10 days' infection.

3. These results were discussed in relation to the effects of diet and gut length on the susceptibility of the host.

Table 1. *The worm burdens, lengths of the small intestine infected in milk diet and control cake diet in mature male mice and* 10- *and* 15*-day-old male mice infected for* 5 *and* 10 *days with fifty infective larvae of* Nematospiroides dubius *per mouse*

Diet	Age (days)	duration of infection (days)	No. of mice used	Average worm burden	't' value	No. of male worms	No. of female worms	't' value	% length of gut infected	't' value
Milk	45	5	8	26·43 ± 3·82	3·46	—	—		26·05 ± 2·75	4·41
Milk	45	10	8	34·57 ± 3·01	4·38	15·14 ± 2·0	19·14 ± 1·91	6·78	16·30 ± 1·59	0·01
Cake	45	5	11	12·18 ± 1·42	2·43 15·41	—	—		26·09 ± 2·59	0·92 4·63
Cake	45	10	3	28·67 ± 1·78		12·67 ± 1·44	16·0 ± 0·71	2·64	15·52 ± 1·27	
Suckling	10	5	3	19·33 ± 0·67	0·11	—	—		68·03 ± 14·53	0·49
Suckling	10	10	3	19·0 ± 3·06	4·3	9·67 ± 0·33	9·30 ± 3·38	0·13	61·37 ± 4·87	1·02
Cake	15	5	6	36·5 ± 4·05	7·77 0·02	—	—		53·75 ± 3·91	10·33 11·55
Cake	15	10	7	36·57 ± 2·06		18·42 ± 1·39	18·14 ± 1·25	0·46	16·43 ± 2·20	

This work was done during the tenure of a Department of Scientific and Industrial Research Studentship. My thanks are due to Dr E. T. B. Francis for his helpful and critical supervision, and to Professor I. Chester Jones, in whose department the work was done, for the facilities he provided.

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Demodex aurati sp.nov. and *D. criceti*, ectoparasites of the Golden Hamster (*Mesocricetus auratus*)

By Wm. B. NUTTING
University of Massachusetts, Amherst, Massachusetts

(*Received* 18 *February* 1961)

Hirst (1918) first reported the presence of two distinct species of the genus *Demodex* from a single host species. These were *Demodex ratti* Hahn and *D. nanus* Hirst from the brown rat, *Rattus norvegicus*. This same observation appeared as a note in his excellent monograph of the genus in 1919. In this latter work Hirst stated that *D. nanus* was probably synonymous with *D. soricinus* Hirst, 1918. He also (Hirst, 1921) mentioned the marked variability of specimens in his description of *D. gliricolens*. He noted that both short and elongate specimens of this species were present on *Arvicola amphibus*. Bennison (1943) recorded two species, *D. folliculorum* var. *equi* and *D. equi*, from the horse, *Equus caballus*. Nutting & Rauch (1958) in a description of *D. criceti* noted the presence of an elongate member of this genus on *Mesocricetus auratus*.

The following report is concerned with a study of this new species, *D. aurati*, and a comparison of its external morphology and distribution with *D. criceti*.

Demodex aurati sp.nov. (all measurements in microns)

Description (including characters of the genus): *D. aurati* is a very thin elongate member of the genus. Adults quite variable in length. Opisthosomae terminally acute in all stages of life cycle.

Male (Pl. 1, fig. 1): Mean body length for twenty specimens 183·1 ± 14·5; about 126 of this opisthosoma. Other measurements in Table 1.

Capitulum (gnathosoma) trapezoidal in dorsal aspect. Its length almost identical to width at base. Tip of capitulum (span of palps plus chelicerae) slightly more than one-half width of base. Capitular spines short (*ca.* 4), thin and slightly curved (Text-fig. 5) with distal portion pointed mediad. Basal peduncle small, largely obscured by proximal portion of the distal segment. Five very small setae clumped on anterior face of each palp.

Four pairs of legs, evenly spaced on podosoma and clearly visible from above.

Genital orifice non-operculate, opening dorsally at a level posterior to a line between second pair of legs and slightly anterior to the posterior dorsal tubercles. Anterior dorsal tubercles slightly anterior to a transverse plane midway between first and second pairs of legs. Penis approximately 15·6 in length and 1 in width, and appears to possess a terminal sheath (Text-fig. 3). Several faint striae parallel the long axis of the penis on the dorso-lateral podosoma. More prominent transverse striae present directly above last pair of legs.

Table 1. *Means and standard deviations in measurements of twenty specimens each of* Demodex criceti *and* D. aurati. *All measurements in microns*

	*D. criceti**	*D. aurati*
	Female	
Capitulum: length	15·4 ± 0·9	18·2 ± 1·6
width	19·8 ± 1·7	16·8 ± 0·9
Podosoma: length	41·6 ± 1·9	45·1 ± 2·1
width	33·3 ± 1·3	25·0 ± 1·1
Opisthosoma: length	46·1 ± 2·9	128·6 ± 19·0
width	33·6 ± 0·6	22·7 ± 1·2
Total length	103·4 ± 2·5	191·9 ± 21·0
	Male	
Capitulum: length	14·7 ± 1·1	15·8 ± 0·7
width	17·0 ± 1·1	16·0 ± 0·9
Podosoma: length	42·2 ± 1·8	41·0 ± 1·2
width	31·6 ± 1·2	21·1 ± 1·2
Opisthosoma: length	30·5 ± 3·8	126·2 ± 14·1
width	32·2 ± 0·6	20·5 ± 1·2
Total length	87·4 ± 4·0	183·1 ± 14·5
	Ovum	
Length	37·9 ± 2·5	64·7 ± 1·2
Width	20·3 ± 1·7	16·6 ± 1·1
	Larva	
Length	55·9 ± 2·5	104·0 ± 16·0
Width	28·8 ± 3·3	17·2 ± 2·1
	Nymph	
Length	84·4 ± 10·7	206·9 ± 25·1
Width	30·5 ± 1·7	24·7 ± 3·1

* From Nutting & Rauch, 1958.

Posterior epimeral plates bounded behind by a distinct suture.

Female (Pl. 1, fig. 2). Mean body length for twenty specimens 191·9 ± 21; about 128 of this opisthosoma. Other measurements in Table 1.

General shape of capitulum as in male except that slightly longer than wide. Span of palps and chelicerae 11·2. Capitular spines and palpal setae identical to those of male.

General contours of podosoma, podosomal striations and disposition of legs identical to male. In both podosomal length and width female averages *ca.* 4 larger than male (Pl. 1, fig. 2).

The vulva lies in a short triangular plate bounded by posterior epimeral plates and the first segmental suture of opisthosoma (Text-fig. 1). Vulva usually tadpole-shaped with enlarged portion anteriorly. It measures 8·6 long × 1·6 at greatest width.

Distinct sutures separate the posterior epimeral plates from opisthosoma and genital plate.

Ova. Spindle-shaped, thin-shelled, and transparent. Length about four times width. The end of the egg which will become the posterior of adult is acute (Pl. 1,

fig. 3). A pigment spot, present in all stages of the life cycle, appears in this posterior portion. Means with standard deviations for measurements of twenty ova are: length $64{\cdot}7 \pm 1{\cdot}2$; width, $16{\cdot}6 \pm 1{\cdot}1$.

Larvae. Attenuated and quite variable in length (Pl. 1, fig. 5). Means with standard deviations for measurements of twenty specimens: length $104{\cdot}0 \pm 16$; width $17{\cdot}2 \pm 2{\cdot}1$.

Leg plates six in number, each with two minute trifid claws. Six epidermal scales present. These long simple sinuous shelves with minute indentations in medial anterior margin. Mouthparts well developed.

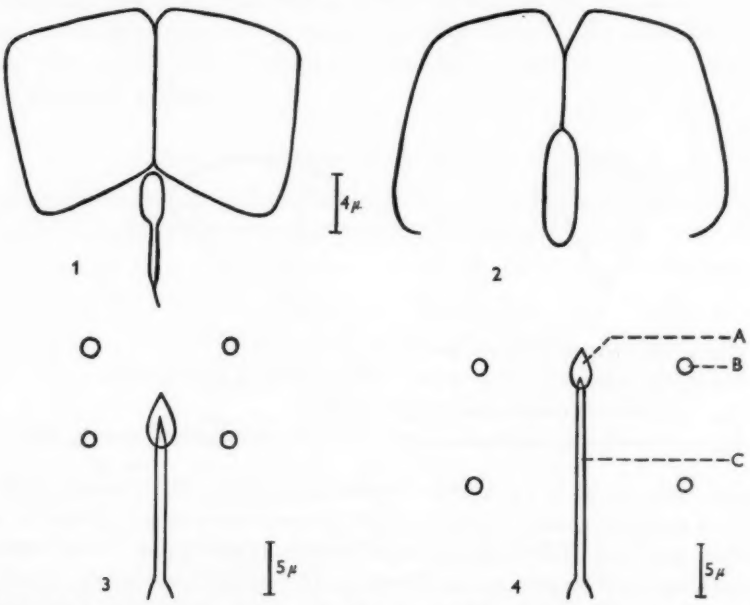

Fig. 1. Tadpole-shaped vulva showing relationship to epimeral plates in *D. aurati*.

Fig. 2. Vulva of *D. criceti* showing relationship to epimeral plates.

Fig. 3. Penis of *D. aurati* showing relationship to dorsal tubercles.

Fig. 4. Penis of *D. criceti* showing relationship to dorsal tubercles. Genital orifice (A), dorsal tubercle (B), and penis (C). From Nutting & Rauch, 1958.

Nymph. Specimens very variable in length. No sure criteria yet known to separate deutonymphs from protonymphs. Means with standard deviations for measurements of twenty specimens: length $206{\cdot}9 \pm 25{\cdot}1$; width $24{\cdot}7 \pm 3{\cdot}1$.

Nymphs (Pl. 1, fig. 7) relatively thick bodied with very attenuated 'tails'—in general contours much like nymphs of *D. caprae* (Cram, 1925). Eight leg plates present, each bearing two minute trifid (?) claws. Eight epidermal scales present, each with basal plate. Long triangular processes extend posteriorly from the margins of the posterior three pairs of scales. Scales measure 2–3·5 (Text-fig. 7).

Diagnosis. *D. aurati* most closely resembles *D. nanus* Hirst (probably, according to Hirst, 1919, synonymous with *D. soricinus*). It differs from this species in

that the capitular spines are well developed in *D. aurati*, but minute or wanting in *D. nanus;* female capitulum, podosoma, and opisthosoma of *D. aurati* are longer than these parts in *D. nanus*. Measurements for *D. nanus* (Hirst, 1919) are: capitulum 13–14; podosoma 34–35; and opisthosoma 39–55.

Unfortunately the ova, larvae, and nymphs of *D. nanus* are unknown.

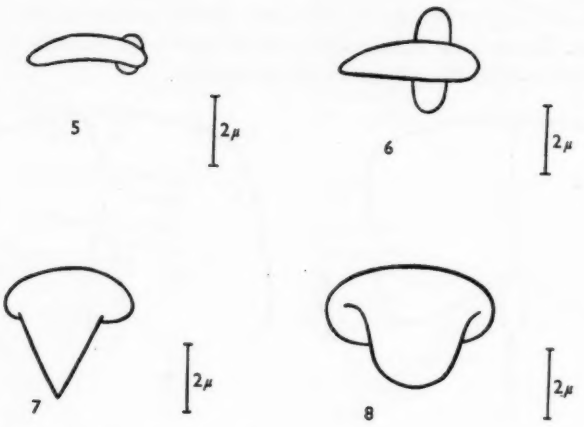

Fig. 5. Capitular spine of *D. aurati*.

Fig. 6. Capitular spine of *D. criceti*. From Nutting & Rauch, 1958.

Fig. 7. Posterior epimeral scales of *D. aurati*.

Fig. 8. Posterior epimeral scales of *D. criceti*. From Nutting & Rauch, 1958.

TYPES. Slide no. 30 in the author's collection contains the following specimens designated as types: a male (ringed in black) as holotype, a female (ringed in white) as allotype, and two other males and eight females as paratypes. Slides with paratype specimens will be sent to the Museum of Comparative Zoology, Cambridge, Massachusetts; U.S. National Museum, Washington, D.C.; British Museum of Natural History, London, England; and the Chicago Academy of Sciences, Chicago, Illinois.

HOST. *Mesocricetus auratus*, the Golden Hamster. All specimens for this description were obtained by Dr Miriam Shaw and the author from laboratory strain N-1 in 1958.

PATHOLOGY. *D. aurati* is found in the hair follicle where it appears to be harvesting the cells of the follicular epithelium. Occasionally adults are found with their mouthparts directed into the sebaceous gland. Destruction of sebaceous cells directly adjacent indicate that these are also eaten by the mites.

Adult mites and ova are found in nearly all instances at or above the opening of the sebaceous glands. Larvae and nymphs found deep in the hair follicle appear to have their mouthparts penetrating cells—often even below the clubs of resting hairs (telogen phase). Sections of heavily infested skin (as many as five mites per hair follicle) show no gross evidence of mite activity. Pigment spots commonly found in the epidermis of aged hamsters are apparently not related to mite activity

since they do not coincide with the positions of the hair follicles—habitat of the mites.

LIFE CYCLE. The eggs of *D. aurati* are laid singly in the hair follicle at or above the level of the outlet of the sebaceous gland. Sections of the eggs indicate that they are deposited prior to embryonation. Ensuing stages conform in general sequential characteristics, although differing in external morphology, to those reported for *D. criceti* (Nutting & Rauch, 1958). The very pronounced blades on the epidermal scales of the nymphs appear to be the most important holdfast structure in this stage since the 'leg' tubercles are minute. Both larvae and nymphs are usually found deep in the hair follicle below the level of the sebaceous glands.

Both males and females of *D. aurati* are often found in the same hair follicle. Despite this, no evidence of copulation has been found in our examination of several thousand sections.

Comparison of Demodex aurati *with* D. criceti

EXTERNAL MORPHOLOGY. In addition to the meristic data supplied in Table 1, the following marked differences occur in the two species.

Structure	*D. aurati*	*D. criceti*	Figures	
Ova	Spindle-shaped	Broadly oval	3, 4	Pl. 1
Larva	More attenuated	—	5, 6	Pl. 1
Leg plates	More oval; smaller claws			Pl. 1
Nymphs	Elongate, slender	Stout	7, 8	Pl. 1
Opisthosoma	Tail-like	Rounded to truncate		
Posterior epidermal scales	With large triangular processes	Simple rounded shelves	7, 8	Text-figs.
Adults				
Capitular spines	Elongate; peduncles small	Stout; peduncles large	5, 6	Text-figs.
♂ genital orifice	Anterior to posterior tubercles	Between anterior tubercles	3, 4	Text-figs.
Vulva	Tadpole-shaped	Rectangular	1, 2	Text-figs.
Longitudinal podosomal striae	Present	Absent		
Palpal setae	Small; of same size	Two outer markedly larger		

DISTRIBUTION. *D. criceti* (Nutting & Rauch, 1958) is found in all stages of its life cycle in pits in the host epidermis. These pits are approximately the size of the mites. Specimens which have been found in the epithelia of abnormally distended hair follicles are also in pits with their long body axes perpendicular to the hair shaft. *D. aurati*, on the other hand, is normally found in all stages anterior end down in the hair follicle (Pl. 1, fig. 9) with their long body axes parallel to the hair shafts. On rare occasions adult mites of both species have been observed free upon the host epidermis or under loose slips of epithelial debris.

Ecological isolation for these two species is apparently, then, assured at least during feeding and reproduction (see above and Nutting & Rauch, 1958).

In a study of the differential distribution of seven body areas (eye-lid, muzzle, dorsal head, mid-dorsal, ventral, genitalia, dorsal spot) in sixteen hamsters 20–80 days of age no evidence was found for a gross preferential site on the host anatomy. In senile hamsters (*ca.* 2 years), however, *D. aurati* far out-numbers *D. criceti* especially on the dorsum. This may well be a function of the heavy keratinization of the epidermis (see Nutting & Rauch, 1961) presenting a physical barrier to increase of the latter.

On application of heat to excised skin both species have left their respective habitats. They then wander across the surface of the skin. Further experiments are under way in our laboratory to try to find the importance of this temperature response in the host.

Effect of the environment on Demodex aurati *and* D. criceti

The marked similarity and relatively low standard deviations (Table 1) for the podosomae of both these species of *Demodex* suggested that possibly some environmental influence might be responsible for the differences in ova, larva, nymphs, and opisthosomae of the adults. Neumann (1914) stated that elongate and short forms of *Falciger rostratus* were directly related to the degree of skin moisture.

In an effort to answer this question sections of skin infested preponderantly with *D. aurati* and others preponderantly with *D. criceti* were introduced subdermally in young hamsters. These were sampled periodically—one portion of each sample examined fresh, and the other sectioned and stained.

Despite rapid infiltration of leucocytes and partial breakdown of both epidermal and dermal elements adults of both forms remained alive and unchanged for over 100 days. While there is no assurance that these mites completed their life cycles under these conditions, the time span of the experiment was four to five times that in which isolated young hamsters show very heavy *Demodex* populations—presumably due to mite reproduction. Both ova, larva and nymphs of both species found in these skin subplants (100+ days) were readily assigned to the appropriate species. In all stages, therefore, no detectable morphological difference was occasioned in either species by the change in ecological conditions.

Furthermore, drastic biotin deficiency (100 days) and subdermal transplants in biotin-deficient hamsters had no effect on the morphological characteristics of either *D. aurati* or *D. criceti* (Nutting & Rauch, 1961).

DISCUSSION

The results of our study confirm and extend the observations of Hirst (1919) and Bennison (1943) that in the genus *Demodex* two distinct species may be present on the same host.

Both of these workers based their reports on very small numbers of specimens and therefore left open the possibility that intergrade forms might exist. Hirst, working on specimens obtained by skin scrapes, had no information on ecological isolation of *D. ratti* from *D. nanus*. Bennison reported gross preferential sites which differed for *D. folliculorum equi* (muzzle) and *D. equi* (flanks), but no isolation such as that here detailed for *D. criceti* from *D. aurati*.

While the morphological details used by these authors are apparently valid criteria for species discrimination the present report is the first attempt to assess environmental change as conditioner of such characters in the genus *Demodex*.

Information to date would seem to indicate strong species stability in the genus *Demodex* as contrasted with such mites as *Sarcoptes* (see Baker & Wharton, 1952). However, in view of the peculiar host affinities of the species of *Demodex*, as shown in Table 2, species specificity for this group appears questionable.

Evidence along this line is decidedly unsatisfactory despite reports (as Maynard, 1922) that *D. canis* of *Canis familiaris* is directly transmissible to *Homo sapiens*. In reports of such transmission no adequate measures have been taken to remove the endemic demodicids from the recipient nor have taxonomic characters been carefully studied for species discrimination. The one exception to this statement is an experiment by Nutting (1950) in which specimens of *D. criceti* were substituted for *D. caprae* in pustules of the goat. In this case no living *D. criceti* were recovered.

Table 2. *Affinities of demodicids which are reported as parasitizing identical hosts. Decisions on affinities made by the author from a comparison of descriptions and/or plates of Hirst* (1919), *Bennison* (1943), *Nutting* (1950), *and Nutting & Rauch* (1958)

Author	Host	*Demodex* sp.	Species related	Host
Hirst, 1919	*Rattus norvegicus*	*D. ratti* *D. nanus*	*D.* sp. *D. soricinus* (identical?)	*Arteles* sp. *Sorex araneus castaneus*
Bennison, 1943	*Equus caballus*	*D. equi* *D. folliculorum equi*	*D. bovis* *D. folliculorum*	*Bos taurus* *Homo sapiens*
Nutting & Rauch, 1958	*Mesocricetus auratus*	*D. criceti*	*D. muscardini*	*Muscardinus avellanarius*
This report	*Mesocricetus auratus*	*D. aurati*	*D. nanus* = *soricinus*?	*Rattus norvegicus*

SUMMARY

A new species, *Demodex aurati*, is described from the Golden Hamster, *Mesocricetus auratus*. It differs in both morphological detail and ecological niche from *D. criceti* present on the same host.

When pieces of heavily parasitized skin were 'subplanted' for 100 days no change in morphological features of either *D. aurati* or *D. criceti* occurred.

D. aurati is a low grade pathogen, apparently only harvesting the cells of the follicular epithelium.

The life cycle of *D. aurati* is described.

The presence on one host species of two different species of this genus, which has affinities with demodicids of evolutionally remotely related hosts, poses an interesting problem in species specificity and ectoparasite evolution.

I wish to thank Dr Miriam Shaw, Miss Shirley DiBenedetto and Miss Helen Krause for their excellent technical help, and Dr Lawrence Bartlett and Dr Harold Rauch for perusal of the manuscript and suggestions on project procedure.

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EXPLANATION OF PLATE 1

Figs. 1–8 × 300.

Fig. 1. Male of *D. aurati*. This is an unusually long specimen.

Fig. 2. Female of *D. aurati*. Note vulva situated behind epimeral plates.

Fig. 3. Ovum of *D. aurati*.

Fig. 4. Ovum of *D. criceti*.

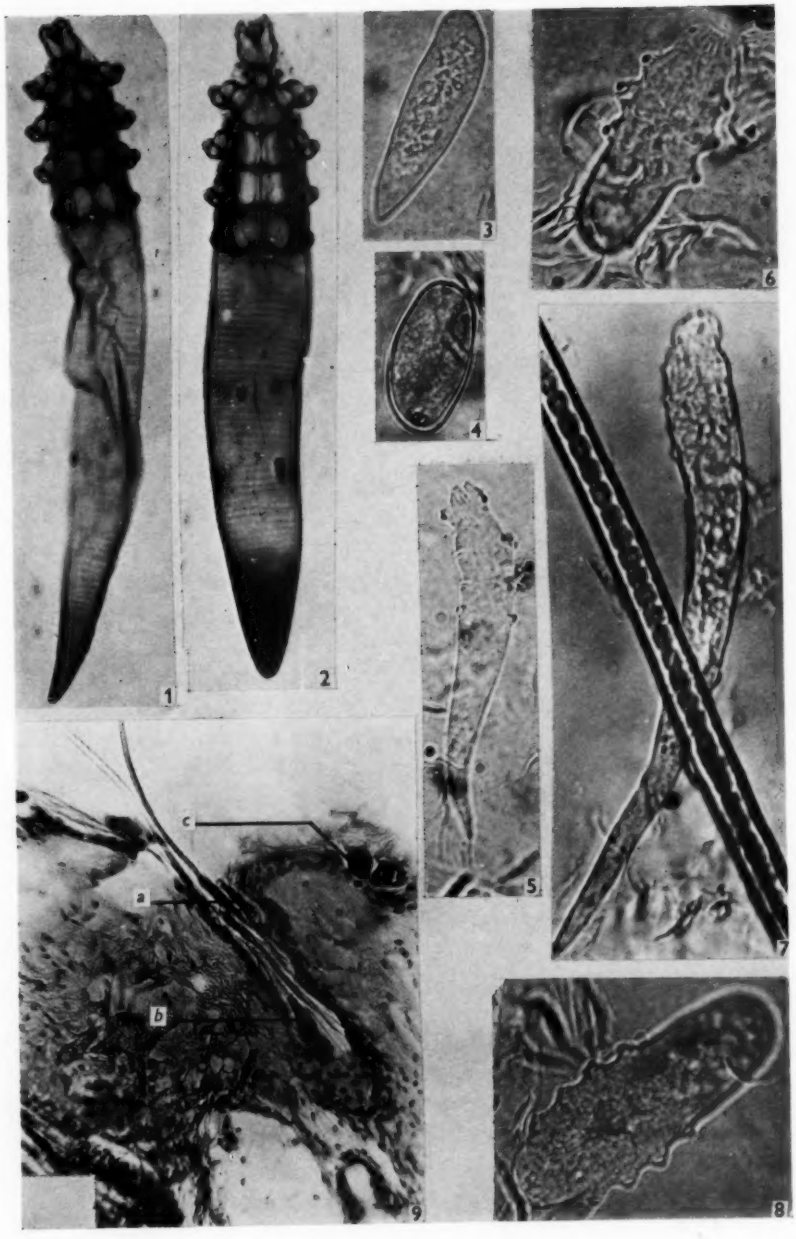
Fig. 5. Larva of *D. aurati*.

Fig. 6. Larva of *D. criceti*.

Fig. 7. Nymph of *D. aurati*.

Fig. 8. Nymph of *D. criceti*.

Fig. 9. Section of skin of *Mesocricetus auratus* showing (*a*) adult of *D. aurati*, (*b*) larva of *D. aurati*, and (*c*) larva of *D. criceti*.



W. B. NUTTING

(*Facing p.* 522)

Parasitology (1961), **51**, 523–532
With 1 plate and 3 text-figures
Printed in Great Britain

Studies on monocystid gregarines

Three *Monocystis* species including two new species *M. cambrensis* and *M. lanceata*

By B. REES

*Forensic Science Laboratory, Jordan Street, Preston**

(*Received* 11 *November* 1960)

INTRODUCTION

The three species of gregarines described in the present paper—*Monocystis cambrensis* sp.nov., *M. lanceata* sp.nov. and *M. elongata* (Phillips & Mackinnon, 1946), Loubatières, 1949—are all parasites of British earthworms. The observations upon which these descriptions are based form part of a larger survey of the monocystid fauna of several British earthworms. The methods used are fully described in a previous paper (Rees, 196).

The genus Monocystis

Although Stein (1848) was the first to use the generic name *Monocystis* for his description of *M. agilis*, it was Hesse (1909) who first described the genus thus: *Body ovoid or spherical without well marked anterior differentiations. Trophozoites solitary.* Cognetti de Martiis (1923) emended the genus in order to separate it from the new genus *Apolocystis*, which he created. This author re-defined it thus: *Trophozoites ovoid without marked differentiation anteriorly, often provided at the anterior pole with a mucron. Solitary.*

The morphology of the species described in the present paper agrees with the definition given by Cognetti.

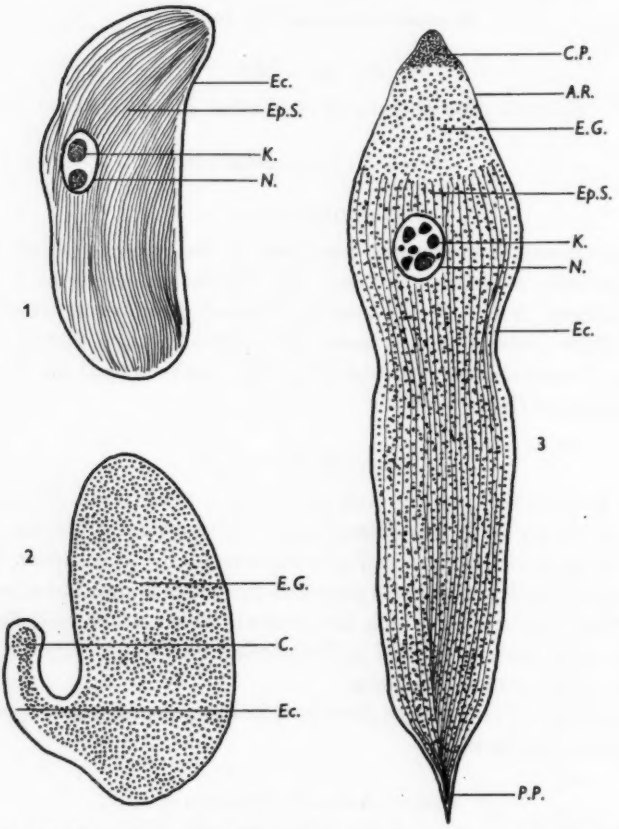
(1) **Monocystis cambrensis** sp.nov.

M. cambrensis is a parasite of *Eisenia foetida*. During the later part of its life-cycle the trophozoites of this species lie free in the body cavity of the worm. The adult trophozoites are extremely active and assume different shapes during different stages of movement. Thus although the most common shape assumed by the trophozoite is that of an elongated slightly curved oval (Pl. 1, fig. 1), it may also assume the shape of an oval with a lateral curved process (Pl. 1, fig. 3) or it may become spherical with a terminal pointed process (Pl. 1, fig. 4). This species may, however, be conveniently described as possessing an elongated, oval-shaped trophozoite with no distinct polarity which may attain a size of 500 μ in its greater diameter.

M. cambrensis has a life-history similar to that of *Cephalocystis singularis* (Rees), in that it has an extended non-migrating tissue phase within the tissues of

* This work was carried out at the Zoology Department, University College, Cardiff.

the gut of the host. During this phase the parasite forms large white cysts on the external surface of the host's intestine (Pl. 1, fig. 5) similar to those formed by *C. singularis*. This non-migrating tissue phase is followed by a period spent free within the body cavity of the host.



Text-fig. 1. Adult trophozoite of *Monocystis cambrensis* showing epicytic striations and nucleus.

Text-fig. 2. Trophozoite of *M. cambrensis* in condition in which it undergoes circular peristaltic contraction.

Text-fig. 3. Adult trophozoite of *M. lanceata*.

Morphology

Ectosarc. The ectosarc is well defined as a clear layer approximately 6 μ thick (Pl. 1, figs. 1–3). The epicyte bears numerous very fine striations which converge to each pole of the animal; critical illumination and focusing are required before these striations become visible. In sectioned material these striations give the epicyte the appearance of a papillated layer approximately 2 μ thick, while the sarcocyte is seen as a faint band up to 2·5 μ thick, whereas the myocyte is not well differentiated.

Endosarc and nucleus. (Text-figs. 1, 2). The endosarc consists of dense granular protoplasm, the granules of which are irregular spheres up to 6 μ in diameter.

The nucleus usually occupies a peripheral position but moves around within the cell body during the extensive movements of the trophozoite. In the living trophozoite the nucleus appears as a large oval vesicle which usually contains two large spherical karyosomes. In a trophozoite 500 μ in its larger diameter the nucleus measures approximately 65×50 μ and usually contains two spherical karyosomes which are slightly dissimilar in size, e.g. 21 and 24 μ in diameter. There is, however, variation in the number of karyosomes which may be present and trophozoites with one or three karyosomes are sometimes found. In fixed and stained specimens, the nuclear membrane shrinks considerably and appears as an irregular membrane surrounding the karyosomes, which are very darkly staining bodies containing numerous small unstaining vacuolar areas.

Movement

M. cambrensis exhibits very characteristic peristaltic movements of two types: lateral and circular. Lateral movement occurs when the body of the trophozoite is evenly expanded in the form of an elongated oval (Pl. 1, fig. 1); it consists of a regular curving and recurving of the whole body of the trophozoite. Starting from rest, the trophozoite first apposes its two poles (Pl. 1, fig. 2), thus assuming an almost spherical shape with a lateral indentation. It then returns to the resting position and recurves in the opposite direction, once more assuming a nearly spherical shape but with the lateral indentation on the opposite side of the trophozoite. This curving and recurving of the trophozoite represents one complete cycle of lateral movement and it takes approximately 6 sec. to complete.

Circular peristaltic movement is characterized by the trophozoite expanding unevenly during the whole of the cycle. During this movement one pole of the trophozoite is permanently contracted, is nearly empty of endoplasm and forms a large curved process at one end of the trophozoite (Pl. 1, fig. 3). Circular peristaltic movement then proceeds by means of a wave of expansion passing around the circumference of the animal, commencing from the concave side of the curved pole of the trophozoite, and proceeding towards the convex side of this pole by passing around the greater bulk of the trophozoite. During a complete cycle of this type, which takes approximately 8 sec. to complete, the curved pole of the trophozoite is thus by-passed and is not involved in the movement.

The formation of the curved pole, prior to the commencement of circular peristaltic activity, is effected by the emptying of the endoplasmic contents of this region into the rest of the body of the trophozoite. In a trophozoite whose overall length is 500 μ the curved process thus formed may be as long as 60 μ. The uneven expansion of the trophozoite during this type of movement is characteristic for the species.

In addition to the diverse shapes that the trophozoite may assume during various stages of its movement, it sometimes assumes a spherical shape with a terminal pointed spike during periods of rest (Pl. 1, fig. 4).

Incidence

The incidence of *M. cambrensis* varied, depending upon the locality from where the worms were collected. It appears to be entirely absent from some areas, whereas in other areas, which are separated by only a few miles, it has a very high incidence. Thus it occurred in 75 % of the worms collected along the banks of the Glamorgan Canal at Whitchurch, Cardiff, while those collected from Roath Park, Cardiff, and from Rhymney, Cardiff, were found to be completely free of this parasite. Both of the latter collecting grounds are separated from the first one by 1 mile and 2½ miles respectively. A similar type of irregular distribution of *Monocystis cognetti* in *Allolobophora chlorotica* has been recorded by Hesse (1909). This author reported finding this species only in specimens of *A. chlorotica* collected from the Botanical Gardens at Grenoble.

Life history

As previously mentioned, *M. cambrensis* spends the earlier part of its life-cycle within the tissues of the intestine of the host until it has attained a comparatively large size (Pl. 1, fig. 5). In this respect it is similar to *Cephalocystis singularis* in having an extended, non-migrating, tissue phase, during which considerable growth takes place. During this phase the parasite forms large white cysts on the surface of the host's intestine from which the trophozoites may be seen to release themselves. The majority of the trophozoites emerge from these cysts when they are between 300 and 400 μ in diameter, although smaller trophozoites have been found free in the body cavity of the host. Active trophozoites can be dissected out from the cysts on the surface of the intestine and these will remain alive in the saline used (Pantin, 1946) for up to 30 hr. After migrating into the body cavity of the host, the trophozoites are very active in their movements and may migrate up into the anterior segments of the worm. Their subsequent behaviour depends upon the presence of other trophozoites in the body cavity. If these are present, association and gametocyst formation can take place although this has not been observed.

The young stages penetrating the mucosa and the muscle layers of the intestine have not been observed in the sections of infected tissue examined. The stages that have been observed, however, are all comparatively large and are contained within a connective tissue capsule which is again surrounded by a layer of peritoneal cells (Pl. 1, fig. 6). In this respect it differs from *Cephalocystis singularis*, which, during its tissue phase, is surrounded by a single cyst wall composed of peritoneal cells, and it is interesting to note that these two species induce different tissue reaction in the same host.

Although the sporozoites and later penetrating stages of this species have not been found migrating through the intestinal mucosa and muscle layers of the host, it is assumed that, owing to its similarity to *C. singularis* in the later stages of its life history, *M. cambrensis* infects the host via the intestinal mucosa before entering on its non-migrating tissue phase between the peritoneum and the intestinal muscle layers.

(2) **Monocystis elongata** (Phillips & Mackinnon, 1946), Loubatières, 1949

This species was first described as *Apolocystis elongata* by Phillips & Mackinnon (1946). Although these workers were mainly concerned with the chromosome cycle of the organism, they gave an account of its morphology and also discussed its taxonomic position. The present brief description mainly reaffirms the description of the original authors, but the correct taxonomic position of the organism is considered.

The original authors found this species parasitizing the seminal vesicles of *Eisenia foetida*. The present description is based upon specimens found in the same host species collected from numerous localities in Glamorgan where it is very common. The incidence of infection of this species varies from 15 to 60 %, but it has been found to occur in all localities where the host has been collected.

Morphology

The trophozoites are elongated oval-shaped organisms (Pl. 1, fig. 7) which are constantly moving. The size of the smallest trophozoite observed was $70 \times 30\ \mu$, whereas the largest was $380 \times 250\ \mu$. The trophozoites at all stages are covered over their whole surface with short, stiff ectoplasmic processes which vary between 3 and 6 μ in length. Phillips and Mackinnon recorded trophozoite sizes of $103 \times 33\ \mu$–$220 \times 95\ \mu$.

Ectosarc. This region of the animal is well defined and may be 7 μ thick in the larger specimens. The sarcocyte constitutes the greater portion of the ectosarc, while the epicyte, as a result of bearing the ectoplasmic processes, is thin and indiscernible as a continuous membrane. These ectoplasmic processes (Pl. 1, fig. 16) consist of protrusions from the epicyte alone and do not include any other part of the ectosarc. They remain the same length throughout the life of the organism. The myocyte is not distinguishable as a definite layer, and although this species exhibits constant movement no myonemes have been observed in either living, or fixed and stained specimens.

Mucron. All the trophozoites of *Monocystis elongata* possess a small conical mucron at one pole (Pl. 1, fig. 16). Phillips & Mackinnon mentioned this structure but did not illustrate it. The mucron varies between 3 and 6 μ in length and consists of a simple protrusion of the ectosarc. Although it is sometines difficult to see the mucron, owing to the continuous movement of the trophozoite, it is present in all specimens.

Endosarc. The endosarc is homogeneous and is packed with endoplasmic granules. In trophozoites between 70 and 100 μ long these granules appear as small, elongated, flake like granules up to 2 μ in length. The granules increase in size with increasing size of the trophozoite. In trophozoites which are longer than 120 μ, the endoplasmic granules become spherical and attain a diameter of 7 μ. Although the trophozoites may grow to 380 μ in length, the endoplasmic granules do not increase beyond 7 μ in diameter.

Nucleus. The nucleus is spherical and moves around within the cell body during

the movements of the trophozoite. Phillips & Mackinnon gave a detailed account of the nucleus and it need only be mentioned that in the living trophozoite it may attain a diameter of 45 μ in the larger specimens. A nucleus of this size contains a large karyosome approximately 15–20 μ in diameter (Pl. 1, fig. 15); in young trophozoites 70–100 μ in length the nucleus is approximately 15–20 μ in diameter.

Movement

M. elongata exhibits continuous movement which is peristaltic, and is of three types: (1) bipolar, (2) unipolar, and (3) circular. The terms bipolar and unipolar are used here for the first time to describe special types of peristaltic movement.

In bipolar peristaltic movement, a wave of contraction commences at one pole of the trophozoite and proceeds to the other pole (Pl. 1, fig. 17) before returning immediately to the pole from which it first started. The whole effect of this type of movement gives the impression of the trophozoite pumping itself backwards and forwards on either side of a fixed point. This movement is very rapid, and a complete cycle, i.e. the passage of the wave of contraction twice along the length of the trophozoite, takes approximately 2 sec. Although this is a very active type of movement, it does not result in any forward progression of the trophozoite.

Unipolar peristaltic contraction leads to forward progression of the trophozoite. During this movement the wave of contraction commences at one pole (Pl. 1, fig. 13) and proceeds to the opposite pole of the trophozoite, where it appears to become absorbed into the body of the trophozoite and does not retrace its course as it does in bipolar peristaltic movement. The movement is then repeated from the same pole that underwent the first contraction. The series of contractile waves produced in this way all pass in the same direction from one pole to the other. This type of movement is initiated from one pole only and it results in a forward progression of the trophozoite, with the pole from which the movements are initiated leading. Unipolar peristaltic movement is somewhat slower than bipolar peristaltic movement, each cycle taking approximately 4–5 sec. for completion; it results in the trophozoite moving along in a series of jerks.

Circular peristaltic movement is exhibited by the larger trophozoites. In this type of movement one or more zones of local contraction pass continually around the circumference of the trophozoite and result in distorting the surface of the animal (Pl. 1, fig. 8). These waves of local contraction are accompanied by a thickening of the ectosarc, and these areas of thickened ectosarc pass around the surface of the trophozoite, marking the progression of the zone of contraction.

Life History

M. elongata passes the whole life-cycle free in the seminal fluid of the host. It does not at any stage penetrate the developing sperm morulae. The method whereby it reaches the seminal vesicles is not known.

Taxonomic position

Phillips & Mackinnon (1946) placed this species in the genus *Apolocystis*, although realizing that the elongated shape, which this species exhibits, con-

tradicted the morphological definition of the genus *Apolocystis*. They placed it in this genus because its shape and movement superficially resemble those of *Apolocystis gigantea* Troisi (1933). The movement of the latter species, however, is unlike the typical movement of *Monocystis elongata* and resembles the circular peristaltic movement which is characteristic of most *Apolocystis* species.

Monocystis elongata does resemble species of the genus *Apolocystis* in various stages of its movement, and in the nearly spherical shape that it sometimes assumes. The presence of the mucron, however, gives the trophozoite a definite polarity, which, if the definition of the genus *Monocystis* is to be rigidly interpreted, differentiates it from the genus *Apolocystis*. Loubatières (1949) suggested that this species should be transferred to the genus *Monocystis*, but he did not give his reasons supporting this. I believe that the elongated shape of this species, together with the possession of the mucron which gives the trophozoite a distinct polarity, necessitates its transfer to the genus *Monocystis*.

(3) **Monocystis lanceata** sp.nov.

M. lanceata is a rare parasite which has been found in 7½ % of all the specimens of *Allolobophora caliginosa* obtained from several collecting grounds in the Cardiff area. The trophozoites occur free in the body cavity of the host where they are found restricted to the segments in the posterior two-thirds of the worm. It has an elongated pear-shaped trophozoite, which is rounded anteriorly and terminates posteriorly in a sharp point (Pl. 1 , fig. 9, Text-fig. 3). This posterior attenuated region is constant in form and position, and gives the trophozoite a distinct polarity. *M. lanceata* is a relatively inactive species and the largest trophozoites may attain a length of 470 μ and a width of 90 μ. The smallest trophozoite found measured 180×45 μ.

Morphology

Ectosarc. The ectosarc consists of a clear cytoplasmic zone, up to 7 μ thick, which is clearly defined in the posterior two-thirds of the trophozoite, while in the anterior region it becomes very thin, and is indiscernible over the anterior eighth of the cell body (Pl. 1, figs. 9, 10). The posterior pointed region of the trophozoite, which may reach a length of 15 μ in the larger specimens, is nearly entirely composed of ectosarc. The sarcocyte makes up the greater portion of the ectosarc, while the epicyte, which is very thin, bears numerous very fine striations. These striations merge towards the posterior pole but are not present on the anterior eighth of the trophozoite. The myocyte is indistinguishable as a distinct layer.

Endosarc and nucleus. The endosarc is heavily granular, containing large oval granules which may reach 8 μ in their longer diameter in the larger trophozoites.

The nucleus is spherical; it is normally situated in the anterior third of the trophozoite (Pl. 1, fig. 10) and contains numerous small karyosomes (Pl. 1, fig. 14). In the largest trophozoites the nucleus may attain a diameter of 45 μ, while the contained karyosomes may reach a size of 12 μ. In fixed and stained trophozoites the nucleus appears to contain a large, darkly staining, irregular shaped chromatic mass.

Movement

M. lanceata is a relatively inactive slowly moving species, it does, however, exhibit two distinct types of movement: (1) peristaltic and (2) amoeboid. The peristaltic movement as exhibited by this species is a very slow movement. It consists of a slow dilatation of the anterior region of the trophozoite, which is caused by a contraction in length of the whole cell body, forcing the endoplasmic granules from the posterior region forward into the anterior region. During this dilatation of the anterior region of the trophozoite, the diameter of the posterior region remains the same. The movement is completed by a contraction of the anterior region, which now decreases in diameter, forcing the endoplasmic granules back into the posterior region. Thus during this peristaltic movement the trophozoite decreases in length, and the concomitant increase in diameter nearly all occurs in the anterior third of the cell body.

The second type of movement exhibited is very unusual and characteristic for this species, and only the anterior third of the trophozoite is involved during its performance. As previously mentioned, the ectosarc becomes very thin over the anterior region, while both the ectosarc and the epicytic striations become indiscernible over the anterior eighth of the trophozoite. This appears to be a modification of amoeboid movement, as it is this region which is the centre of this type of movement. The anterior eighth of the body of the trophozoite does not seem to be differentiated into endosarc and ectosarc, but forms an anterior 'cytoplasmic plug' which appears to be vacuolar. Amoeboid movement, which results in forward progression during which the trophozoite appears to 'glide' along, commences with a protrusion from the small anterior cytoplasmic plug (Pl. 1, fig. 11). Once this protrusion is formed, the remainder of the modified anterior region increases in diameter and appears to flow into the protrusion, 'dragging' the whole of the trophozoite after it in a slow gliding motion, with the anterior end leading (Pl. 1, fig. 12). During this type of movement, a trophozoite 200 μ long can move along its own length in approximately 65 sec.: the larger trophozoites do not exhibit this amoeboid movement.

Life history

The smallest trophozoites of this species that have been observed were all found in the body cavity of the host. The mode of entry of the parasite is not known nor is anything known about its earlier life history; similarly, association of gametocytes and gametocyst formation have not been observed.

KEY TO LETTERING OF TEXT-FIGURES

A.R.	Anterior region in which the ectosarc becomes indistinct.	*E.G.*	Endoplasmic granulation.
C.	Curved process formed during circular peristaltic contraction.	*Ep.S.*	Epicytic striations.
C.P.	Cytoplasmic plug.	*K.*	Karyosome.
Ec.	Ectosarc.	*N.*	Nucleus.
		P.P.	Posterior pole.

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EXPLANATION OF PLATE 1

Fig. 1. Adult trophozoite of *Monocystis cambrensis* prior to commencement of lateral contraction. × 80.

Fig. 2. Trophozoite of *M. cambrensis* undergoing lateral contraction. The poles of the trophozoite are apposed enclosing a hemispherical lateral indentation. Note well defined ectosarc. × 80.

Fig. 3. Trophozoite of *M. cambrensis* at commencement of circular peristaltic movement. Note the uneven expansion causing the formation of a long curved process at one pole. × 110.

Fig. 4. Trophozoite of *M. cambrensis* at rest having assumed a spherical shape. Note terminal pointed spike. × 140.

Fig. 5. Section through intestine of *Eisenia foetida* infected with *M. cambrensis*. Note two trophozoites contained within cysts formed by the host tissue. × 70.

Fig. 6. Enlargement of cyst containing *M. cambrensis* on intestine of *E. foetida* showing the double nature of the cyst wall. × 190.

Fig. 7. Young trophozoite of *M. elongata*. The mucron is just visible at one pole. Note the covering of short ectoplasmic processes. × 405.

Fig. 8. Mature trophozoite of *M. elongata* undergoing circular peristaltic movement. Note three zones of local contraction with regions of thickened ectosarc. × 360.

Fig. 9. Trophozoite of *M. lanceata* in resting condition. Note posterior pointed pole and the thin ectosarc in the anterior region. × 420.

Fig. 10. Trophozoite of *M. lanceata* undergoing peristaltic movement. Note the dilatation of the anterior region which contains the nucleus. × 400.

Fig. 11. Trophozoite of *M. lanceata* undergoing first stage of amoeboidal movement. Note the protrusion from the anterior region of the trophozoite. × 415.

Fig. 12. Trophozoite of *M. lanceata* undergoing second stage of amoeboid movement. Note the increase in the diameter of anterior region. × 420.

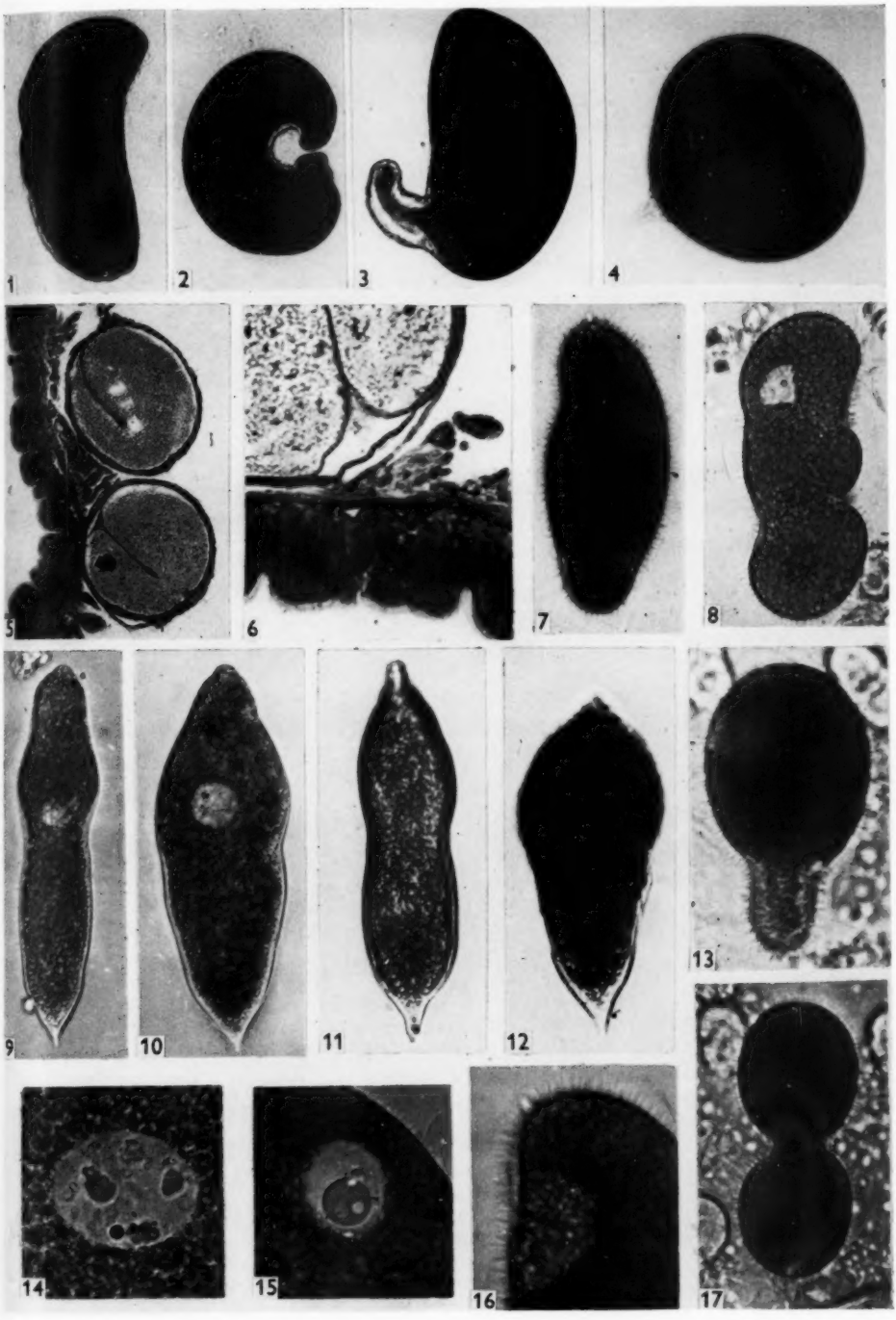
Fig. 13. Trophozoite of *M. elongata* showing commencement of unipolar peristaltic movement. Note the formation of a zone of contraction at one pole. × 460.

Fig. 14. Nucleus of *M. lanceata* containing numerous karyosomes. × 460.

Fig. 15. Nucleus of *M. elongata* containing large spherical karyosome. × 465.

Fig. 16. Anterior pole of *M. elongata* showing small conical mucron which appears light in colour. Note the ectoplasmic processes. × 650.

Fig. 17. Trophozoite of *M. elongata* undergoing bipolar peristaltic movement. The wave of contraction is in the middle of the trophozoite. A similar appearance is produced in the middle of a cycle of unipolar peristaltic movement. × 400.



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Some cyclophyllidean cestodes from carnivores in Southern Rhodesia

By D. F. METTRICK, B.Sc., Ph.D.,†
and MARY BEVERLEY-BURTON, B.Sc., Ph.D., D.I.C.*
Department of Zoology, University College of Rhodesia and Nyasaland, Salisbury, Southern Rhodesia

(*Received* 10 *October* 1960)

The material described in the present paper came from several sources. Mr E. Davison, Chief Game Warden at Wankie National Park, kindly collected the material from a leopard and also provided the hyaena for us to examine. We are also indebted to Mr A. Fraser, Director of Game Conservation, who provided facilities for us to collect material in the vicinity of Lake Kariba where the genet was examined, and to Mr J. Condy of the Veterinary Research Department who shot the serval near Salisbury and brought it to us.

The worms were washed in saline and fixed in formal-acetic-alcohol. The specimens were stained in Kirkpatrick's carmalum. Transverse sections were cut of several proglottids using the polyethylene glycol distearate embedding technique. Sectioned material was subsequently stained in haematoxylin and eosin.

The helminthic fauna of Southern African mammals and birds has, in comparison for example with that of South America and Australia, been neglected. Descriptions of single species collected from Africa are scattered throughout the literature. Baer (1923, 1924) reported on large collections of cestodes, and Ortlepp, between 1933 and 1940, published a series of papers describing South African forms and (1938) described seven new taenioid species from the larger African carnivores. Fain (1952), working in the Congo, elucidated the life cycle of *Taenia brauni* Setti, 1897; Mahon (1954) reported on a diverse collection of cestodes also collected in the Congo; and Baer & Fain (1955) identified the cestodes collected during an exploration of the Parc National de l'Upemba. More recently, Baer (1959) has reported on the helminths collected on his 1958 expedition to the Congo.

The present paper is apparently the first record of taenioid and dilepidid cestodes from mammals in Rhodesia.

* Nuffield Research Fellow in Helminthology.
† Present address: University College of West Indies, Mona, Kingston, Jamaica, West Indies.

CYCLOPHYLLIDEA Ben. in Braun, 1900

DILEPIDIDAE Railliet & Henry, 1909

Dipylidiinae Stiles, 1896

Joyeuxiella Fuhrmann, 1935

Joyeuxiella paucitestis sp. nov. (Fig. 1)

The strobila is small with a maximum length of 20 mm., and a maximum width of 0·9 mm. The scolex (Fig. 1*a*), 0·23–0·25 mm. in diameter, has four well developed, almost round, unarmed suckers, 0·07 mm. long and 0·08–0·09 mm. wide.

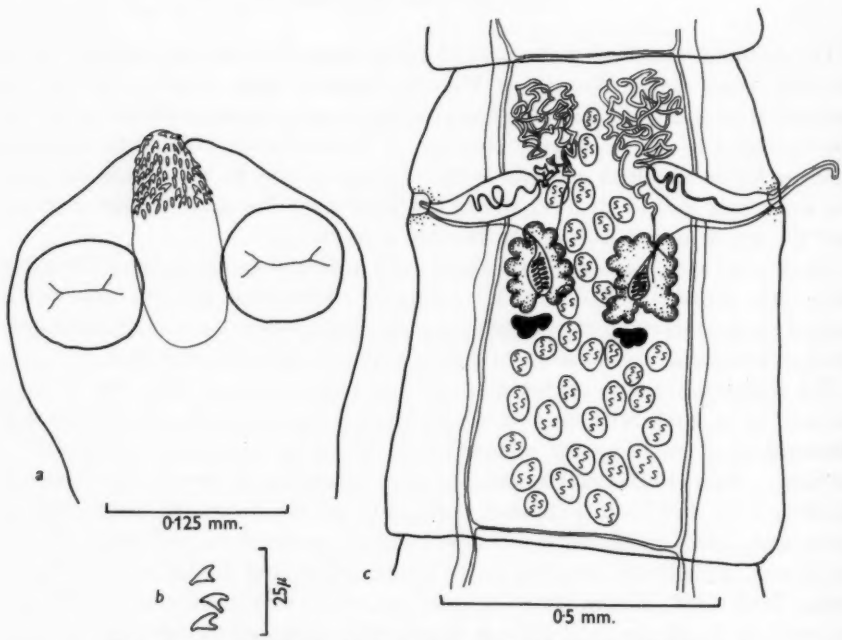

Fig. 1. *Joyeuxiella paucitestis* sp.nov. *a*, Scolex; *b*, rostellar hooks; *c*, mature segment.

The rostellum is conical in shape, 0·05–0·08 mm. long and 0·06 mm. wide at the base. The numerous rostellar hooks are thorn-shaped, 10–12 μ long and are arranged in 10 rows (Fig. 1*b*). The neck, 0·26–0·35 mm. long, has a diameter of 0·18–0·21 mm.

There are two complete sets of genitalia in each segment (Fig. 1*c*). The genital atria open laterally in the anterior third of the segment. The anlagen of the male genitalia first appears in segment 6; the testes are distinct in segment 15. A typical segment in this region is 0·3 mm. long and 0·4 mm. wide. The segments are fully mature by segment 25, and a typical mature segment is 1·1 mm. by 0·7 mm.

There are 31 to 42 testes in each segment, filling the posterior region of the segment and extending anteriorwards between the two ovaries and the coiled vasa

deferentia. The testes are 0·06–0·08 mm. in diameter. The cirrus sac, 0·24–0·25 mm. long by 0·07–0·08 mm. wide, contains a coiled internal seminal vesicle. The cirrus sac runs obliquely upwards from the genital pore and crosses the lateral excretory canals. The cirrus is unarmed, and when extruded measures up to 0·14 mm. long and 17–18 μ in diameter. The convoluted vasa deferentia, 4–5 μ in diameter, lie in the anterior part of the segment, and fill nearly all the available space between the cirrus sacs and the anterior margin of the segment. The vas deferens of each side remains quite discrete. The vagina opens into the genital atrium posterior to the cirrus and curves downwards to the ovary where it dilates to form a receptaculum seminis 0·10–0·12 mm. long and 0·05 mm. wide. The ovary, 0·16–0·19 mm. in diameter, is lobed and almost surrounds the receptaculum seminis. The compact vitellarium is oval, post-ovarian and 0·07–0·08 mm. in diameter.

The gravid segments, typically 0·87 mm. wide and 2·62 mm. long, contain a large number of egg capsules each containing a single egg. The capsules are 0·04 mm in diameter, while the embroyos are 20–24 μ wide.

Host. Genetta rubiginosa. Rusty Spotted Genet.

Location. Intestine.

Locality. Lake Kariba, Zambezi Valley, Southern Rhodesia.

Type to be deposited in the British Museum (Natural History).

Discussion

The present material falls in the genus *Joyeuxiella* Fuhrmann, 1935, which is separated from the other genera in the subfamily Dipylidiinae on differences in the arrangement of the genitalia, and the number of eggs in each egg capsule. Yamaguti (1959) lists nine species in the genus *Joyeuxiella*, omitting the *Joyeuxiella* sp. Kofend, 1917, which Lopez-Neyra (1927) had included in his key.

Witenberg (1932) considered that, of the 10 species listed by Lopez-Neyra, *J. aegyptica* Meggitt, 1927, *J. chyzeri* Ratz, 1897, *J. fuhrmanni* Baer, 1924, *J. pasqualiformis* Lopez-Neyra, 1928, *J. rossicum* Skrjabin, 1923, and Kofend's species were all synonymous with *J. pasqualei* Diamare, 1893. Witenberg also suggested that the species *J. gervaisi* Setti, 1895, and *J. dongolensis* Beddard, 1913, were too briefly described to be acceptable, and that *J. echinorhynchoides* Sonsino, 1889, was the only other valid species in the genus.

This synonymy was accepted by Neveu-Lemaire (1936) and by Hughes, Baker & Dawson (1941). Neveu-Lemaire was apparently under the impression that *J. pasqualei* was the type species of the genus *Joyeuxiella* Fuhrmann, 1935 (nomen novum for *Joyeuxia* Lopez-Neyra, 1927, which was pre-occupied). In fact *J. chyzeri* is the type species, and therefore, unwittingly, Neveu-Lemaire invalidated the genus. (Witenberg had similarly invalidated *Joyeuxia* by his synonymy.) The synonymy proposed by Witenberg (1932) has not been widely accepted by subsequent authors, e.g. Ortlepp (1938), Wardle & McLeod (1952), etc. Therefore, as the type material is not available, the present authors provisionally recognize the species listed by Yamaguti (1959).

The present material may be separated from these nine species by the size and arrangement of the rostellar hooks and the number of testes in each segment.

Joyeuxiella fuhrmanni (Baer, 1924) Fuhrmann, 1935 (Fig. 2)

The strobila is medium in size with a maximum length of 94 mm. and a maximum width of 1·6 mm. The scolex, 0·35 mm. in diameter, has four prominent oval suckers which are 0·09 mm. long and 0·13–0·14 mm. wide. The rostellum, 0·14–0·17 mm. long and 0·09 mm. in basal diameter, is armed with 17–19 rows of thorn-shaped hooks. The hooks of the anterior rows (Fig. 2) are 12–16 μ long, with a basal length of 10–11 μ, but become smaller near the base of the rostellum. The rostellar sac, when the rostellum is extruded, measures 0·16 mm. long by 0·09 mm. wide. The neck is 1·13 mm. long and 0·26 mm. wide. The excretory system is of the normal cyclophyllidean pattern. The ventral excretory canal measures 29 μ in diameter, the dorsal canal 2 μ wide and the transverse canal 5 μ. As is usual in the genus *Joyeuxiella* the genitalia are double in each segment. The anlagen of the male genitalia appears at about segment 75. A typical immature segment in this region is 0·19 mm. long and 0·44 mm. wide. The segments rapidly become mature

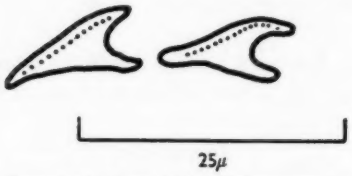

Fig. 2. *Joyeuxiella fuhrmanni* (Baer, 1924) Fuhrmann, 1935. Rostellar hooks.

and a typical mature segment measures 0·52 mm. long by 1·13 mm. wide. The genital atria, 47–50 μ deep, open laterally in the anterior half of the segment. The genital ducts pass inwards from the genital atria, between the lateral excretory canals. There are 33 to 42 testes in each segment. The testes lie behind and between the female genitalia and extend anteriorwards between the vasa deferentia. The testes are 0·05–0·06 mm. in diameter. The coiled vas deferens reaches the anterior border of the segment and has a diameter of 12 μ. The cirrus sac is 0·24–0·26 mm. long by 0·05–0·07 mm. wide when the cirrus is not extruded. The cirrus is unarmed, and measures up to 0·27 mm. long with a diameter of only 0·02 mm. The cirrus sacs do not extend much beyond the level of the excretory canals.

The fan-like ovaries are lateral in position and have a maximum diameter of 0·12–0·16 mm. The vitellaria, 0·04–0·06 mm. in diameter, are compact and lie posterior to the ovaries. The vagina, 16 μ in diameter, opens into the genital atrium behind the cirrus sac. The vagina narrows to a diameter of 7 μ, and then expands to form a large receptaculum seminis. The receptaculum seminis is 0·10 mm. long and 0·05 mm. wide, and is situated among the ovarian lobes. The gravid segments are 4·9 mm. long and 1·6 mm. wide. The egg capsules have a diameter of 54–59 μ, and do not occur lateral to the excretory canals. The embryos are 33–40 μ in diameter.

Host. Zibethailurus serval. Serval.

Location. Intestine.

Locality. Salisbury, Southern Rhodesia.

Discussion

A re-description of *J. fuhrmanni*, based on material recovered from a serval (*Zibethailurus serval*) in the Salisbury district, is included in the present paper because of several differences with the type material described by Baer (1926) (see Table 1).

Table 1

	J. fuhrmanni (Baer, 1926)	*J. fuhrmanni* (present material)
Length	30 mm.	94 mm.
Scolex (diameter)	0·24 mm.	0·35 mm.
Rostellum	0·23 mm. long × 0·1 mm. wide	0·14–0·17 mm. long × 0·09 mm. wide
Rows of hooks	14–16	17–19
Size of hooks	?	12–16 μ
Cirrus sac	0·25 mm. long × 0·05 mm. wide	0·24–0·26 mm. long × 0·02 mm. wide
Nos. of testes	40–50	33–42
Egg capsules	42–46 μ	54–59 μ
Embryo	?	33–40 μ

Witenberg (1932) synonymized *J. furhmanni* with *J. pasqualei* (Diamare, 1893), but Ortlepp (1933) did not accept this synonymy and pointed out the differences in the arrangement of the genitalia in the two forms. Neveu-Lemaire (1936) recognized the synonymy suggested by Witenberg.

The present authors do not accept Witenberg's (1932) synonymy of *J. fuhrmanni* with *J. pasqualei*, and concur with Ortlepp that the two species are distinct.

Diplopylidium Beddard, 1913

Diplopylidium paurodex sp.nov. (Fig. 3)

The single entire specimen is small, measuring 6·16 mm. long and 0·51 mm. in maximum diameter. The scolex, 0·33 mm. in diameter, has four well developed suckers which are 0·09–0·10 mm. wide (Fig. 3*a*). The partly retracted rostellum is 0·12 mm. long and 0·12 mm. wide. There are 54 rostellar hooks which are arranged in three rows. There are 18 hooks in each row, and the rows alternate with each other. The hooks of the anterior row (Fig. 3*b*) are 52–59 μ long, those of the median row (Fig. 3*c*) 47–50 μ, and those of the posterior row (Fig. 3*d*) 33–36 μ. The short neck is 0·18 mm. long and 0·28 mm. wide.

There are two complete sets of genitalia in each segment. The genital pores open laterally in the anterior third of the segment. The anlagen of the male genitalia appears in segment three, and the testes are distinct by segment seven. A typical segment in this region is 0·15 mm. long and 0·35 mm. wide. The total number of segments in the specimen is 16.

A typical mature segment (Fig. 3*e*) is 0·62 mm. long and 0·49 mm. in diameter. There are 20–26 testes in each segment, which fill the posterior region of the segment, and extend forwards between the two sets of female genitalia, and the coiled vasa deferentia. The testes measure 39–43 μ in diameter. The coiled vas deferens is 12–14 μ in diameter. In mature segments one set of male genitalia (excluding the testes) may develop more fully than the other. The cirrus sac,

0·15–0·17 mm. long and 35–39 μ in diameter, contains a coiled internal vesicula seminalis, and extends beyond the lateral excretory canals. The cirrus appears to be unarmed, and has a diameter of 2 μ.

The vagina opens into the genital atrium in front of the cirrus. The vagina crosses the distal part of the cirrus sac, and continues posteriorwards to the ovarian region where it expands to form a pyriform receptaculum seminis. The

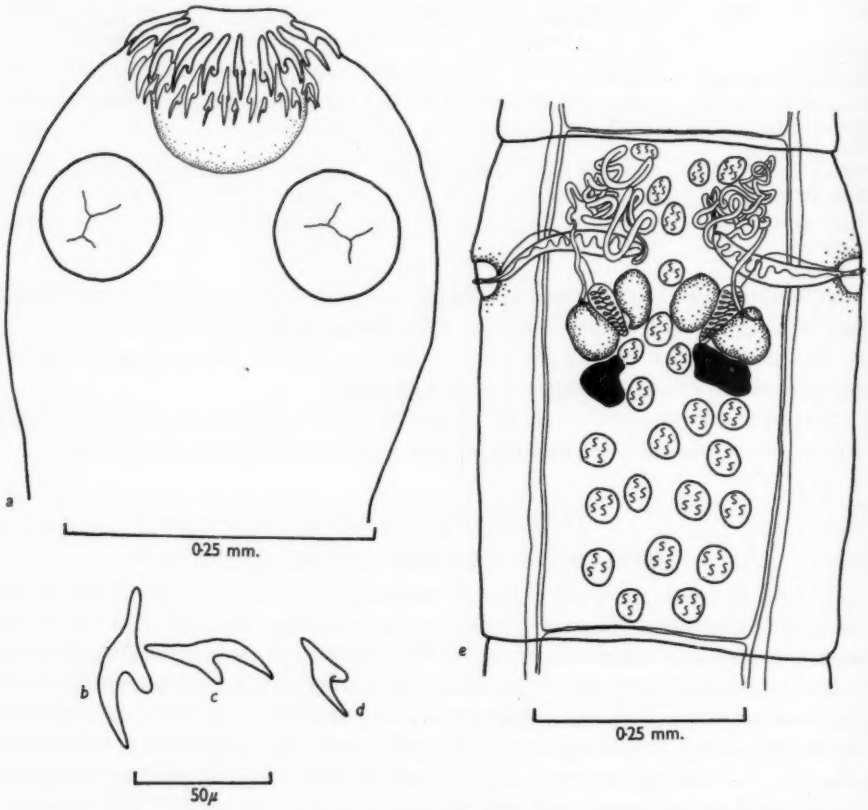

Fig. 3. *Diplopylidium paurodex* sp.nov. *a*, Scolex; *b*, rostellar hook, anterior row; *c*, rostellar hook, median row; *d*, rostellar hook, posterior row; *e*, mature segment.

receptaculum seminis is 82 μ long by 36 μ wide and lies between the two lobes of the ovary. The compact ovary is bilobed and is 0·07 mm. long and 0·14–0·15 mm. in diameter. The vitellarium is immediately post-ovarian, and has a diameter of 0·04–0·05 mm. No fully gravid segments were observed.

Host. Genetta rubiginosa. Rusty Spotted Genet.

Location. Intestine.

Locality. Lake Kariba, Zambesi Valley, Southern Rhodesia.

Type to be deposited in the British Museum (Natural History).

Discussion

The present material was recovered from the same host as the previously described specimens of *Joyeuxiella paucitestis* sp.nov. Although no gravid segments were observed it is placed in the genus *Diplopylidium* Beddard, 1913, because this is the only genus with double genitalia in the sub family Dipylidiinae in which the vagina opens into the genital atrium in front of the cirrus. The generic diagnosis of this genus states that there is a single egg per egg capsule.

Yamaguti (1959) lists eleven species in the genus. But of these, only six have not had their validity questioned. They are *D. genettae* Beddard, 1913 (the genotype which is incompletely described and the type material lost), *D. acanthotretum* (Parona, 1887), *D. monoophorum* (Lühe, 1898), *D. nölleri*, Skrjabin, 1924, *D. skrjabina* Popov, 1935, and *D. zschokkei* (Hungerbühler, 1910). Of the remainder *D. fortunatum* Meggitt, 1927, was considered by Hughes *et al.* (1941) to be a synonym of *J. pasqualei* (Diamare, 1893), while Witenberg (1932) and Neveu-Lemaire (1936) synonymized *D. fabulosum* Meggitt, 1927, *D. trinchesii* (Diamare, 1892), *D. triseriale* (Lühe, 1898) and *D. quinquecoronatum* Lopez-Neyra & Munoy-Medina, 1921, with *D. acanthotretum* (Parona, 1887). Of the 11 species in this genus listed by Yamaguti (1959), the present material appears to be most closely related to *D. zschokkei* (Hungerbühler, 1910) and to *D. acanthotretum* (Parona, 1887). It is distinguished from both these species principally by the number and size of the rostellar hooks, the total size of the worm, the number of testes, and the size of the cirrus sac.

D. acanthotretum has a considerable range in these characters, and it is appreciated that only one entire specimen and several fragments of this new form has been found. Pending the examination of further material, it is considered that this specimen is sufficiently distinct to warrant the erection of a separate species.

TAENIIDAE Ludwig, 1886

Taenia Linnaeus, 1758, s.str. emend. Stiles & Hassall, 1926

Taenia crocutae sp.nov. (Fig. 4)

Several worms were examined and the longest complete specimen observed was over 1,500 mm. in length. As the posterior segments of this specimen were not fully gravid, it is probable that the total length of a gravid specimen would be about 2,000 mm. The maximum width of the strobila was 7·42 mm.

The large scolex, 1·24–1·41 mm. in diameter, has four suckers which measure 0·31–0·32 mm. in diameter. The rostellum, 0·43 mm. in diameter, is armed with 36–40 hooks which are arranged in an alternating double row. The hooks of the anterior row (Fig. 4*a*) are 181–192 μ long, and measure 139 μ along the base. The guard is thick, the blade fairly strongly curved, and the handle more slender than the guard. The hooks of the second row (Fig. 4*b*) are 128–132 μ long and measure 89–90 μ along the base. The guard of these smaller hooks is heart-shaped, when viewed from above, the handle straight, and the blade very strongly curved. The neck is fairly long, and measures about 1·6 mm. long by 0·7 mm. wide. The neck is followed by a long, immature region.

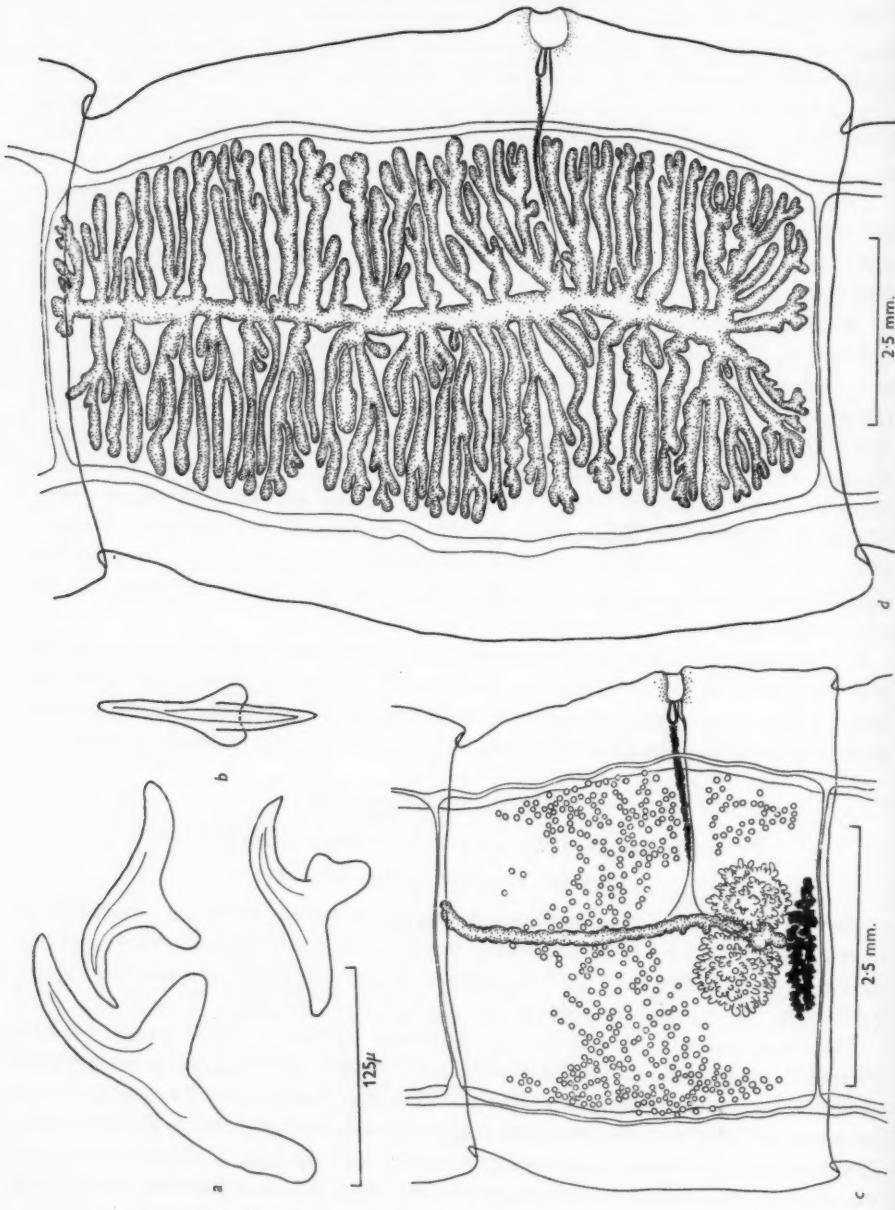

Fig. 4. *Taenia crocutae* sp.nov. *a*, Rostellar hook, anterior row; *b*, rostellar hooks, posterior row; *c*, mature segment; *d*, gravid segment.

A typical mature segment (Fig. 4*c*) measures 7·2 mm. long by 5·1 mm. wide. The genital atria are situated laterally in the posterior half of the segment and alternate irregularly. Each atrium measures 0·32–0·35 mm. long by 0·18–0·20 mm. in maximum basal diameter. The prominent ventral excretory canals are 115 μ in diameter and are linked by transverse canals which are 24 μ wide. The dorsal excretory canals are slender with a diameter of 8 μ. The genital ducts pass between the dorsal and ventral lateral excretory canals, and dorsal to the lateral nerve cord. There are 390–420 testes in each segment. The majority of the testes lie in the aporal half of the segment. A typical segment has 243 aporal and 167 poral testes. The testes, 0·09–0·12 mm. in diameter, do not extend forwards into the anterior region of the segment, nor posteriorly behind the ovary. The coiled vas deferens has a diameter of 24 μ, and passes inwards at right angles with the lateral margin of the segment. The small cirrus sac is pyriform, measures 0·31–0·34 mm. long by 0·12 mm. in diameter and contains coiled internal vesicula seminalis. The cirrus opens into the genital atrium immediately in front of the vagina. The vagina, 16 μ in diameter, passes inwards towards the mid-line before curving towards the posterior part of the segment. The vagina may or may not form a sinuosity before entering the globular receptaculum seminis which measures 0·28 mm. long by 0·14 mm. wide. The ovarian follicles form two hemispherical lobes, which have a total diameter of 2·2 mm. The follicular vitellarium, is postovarian, and is typical of the genus. The vitellarium measures 0·39 mm. long by 1·8 mm. wide. A typical fully gravid segment (Fig. 4*d*) measures 21·4 mm. long by 7·8 mm. wide.

The uterus arises ventrally as a simple tube, extending towards the anterior border of the segment. The uterus forms 19 to 24 primary branches on each side which subdivide forming a total of about 50 lateral branches. The eggs are slightly oval in shape, measuring 36–40 μ long and 28–35 μ wide. The egg shell is thick, and the embryophores are 28–29 μ long and 20–24 μ wide.

Host. Crocuta crocuta. Spotted Hyaena.

Locality. Wankie National Park, S.R.

Location. Intestine.

Type to be deposited in the British Museum (Natural History).

Fig. 4. *Taenia crocutae* sp.nov. *a*, Rostellar hook, anterior row; *b*, rostellar hooks, posterior row; *c*, mature segment; *d*, gravid segment.

Discussion

Using Wardle & McLeod's (1952) preliminary key to the accepted valid species in the genus *Taenia* Linnaeus, 1758, s.str., emend. Stiles & Hassall, 1926, the present material apparently belongs to the same group as *T. ovis* Cobbold, 1860 *T. krabbei* Moniez, 1879, *T. hlosei* Ortlepp, 1938, and *T. hyaenae* Baer, 1926. The last two species are from African hosts.

Both *T. hyaenae* Baer, 1926, and the present species have a characteristic heart-shaped guard on the smaller rostellar hooks which does not occur in the other species of *Taenia* listed above. Baer & Fain (1955) described a new species *T. lycaeontis*, in which the guard of the smaller hooks is bilobed. This, however, appears to be quite distinct from the heart-shaped guard of *T. crocutae* and

T. hyaenae. The blades of the smaller hooks in *T. crocutae* are more strongly curved downwards than those of *T. hyaenae*, also in the former species the handle does not curve upwards and the guard of the larger hooks is less pointed.

Table 2

	Length (mm.)	Nos. of hooks	Size of 1st row (in μ)	Size of 2nd row (in μ)	Nos. of testes	Nos. of main uterine branches	Host
T. ovis	450–1000	24–36	156–188	96–128	300	20–25	*Canis familiaris*
T. krabbei	260	26–34	148–170	85–120	260	10	*C. familiaris*
T. hlosei	325–400	36–40	209–218	142–151	300–400	20–30	*Acinomyx jubatus*
T. hyaenae	300	32–38	196–223	124–156	300	12–14 (sub-divides in rare cases up to 24)	*Hyaena brunea*
T. crocutae sp.nov.	1500+	36–40	181–192	128–132	390–430	19–24	*Crocuta crocuta*

Some confusion appears to have arisen in the literature on the number of primary uterine branches present in *T. hyaenae*. Baer (1926) stated that there were 12–14 primary branches on each side, which in certain abnormal cases subdivided forming 20–24 lateral branches, while Ortlepp (1938) stated that there are up to 26 pairs of *main* uterine branches, and this error is repeated by Wardle & McLeod (1952). Baylis (1937) gave a few notes on some material collected in Tanganyika from spotted hyaenas, which he identified as *T. hyaenae*, although there were 25–28 uterine branches in the gravid segments. He also records that the size of the hooks of the co-type of *T. hyaenae* is 204–212 μ for the large hooks and about 120 μ for the small ones.

Baylis also gives the range in hook size for some specimens recovered from the spotted hyaena in Kenya, in which the size of the large hooks varied between 196 and 204 μ and that of the small hooks between 124 and 130 μ. Baer & Fain (1955) also describe some material (identified as *T. hyaenae*) from a spotted hyaena *Crocuta crocuta* in which the uterine branches number 20–30. It would appear from the table given by Baer & Fain (1955) that the material described by Baylis was all from hyaenas in Kenya. In fact, according to Baylis's original paper, the measurements given by Baer & Fain for the hook size refer to the Kenya material, the other details refer to the Tanganyika material. They suggested that there may be two species involved, separated chiefly by the number of uterine branches, the size of the eggs, the size of the cirrus sac, and possibly the shape of the large hooks.

The present authors concur with this view, this being the third time that material has been found which differs from Baer's original description of *T. hyaenae* by the points listed above. It is suggested that the name *T. crocutae* be given to

the material described above, and that Baylis's and Baer & Fain's material should be assigned to this new species. Table 3 summarizes these descriptions, and compares them with Baer's original description of *T. hyaenae*.

Table 3

	T. hyaenae		*T. crocutae* sp.nov.		
	Baer, 1924, 1926 Type	Baylis, 1937 Co-type	Baylis, 1937	Baer & Fain, 1955	Present material
Length (mm.)	300	—	—	Up to 1000	± 2000
No. of hooks	32–38	—	—	38–40	36–40
Size of 1st row (mm.)	0·223	0·204–0·212	About 0·2	0·19–0·20	0·18–0·19
Size of 2nd row (mm.)	0·127	0·120	0·127–0·156	0·11–0·12	0·12–0·13
Cirrus sac (mm.)	0·4	—	—	0·02–0·25 × 0·07–0·08	0·31–0·34 × 0·08–0·11
No. of testes	300	—	—	400–600	390–420
Nos. of main uterine branches	12–14	—	25–28	20–30	19–24
Size of eggs (μ)	29 × 34	—	28–32 × 32–40	32–35 × 40–43	28–35 × 36–40
Host	*Hyaena brunea*, South Africa	—	*Crocuta crocuta*, Tanganyika	*C. crocuta*, Belgian Congo	*C. crocuta*, Southern Rhodesia

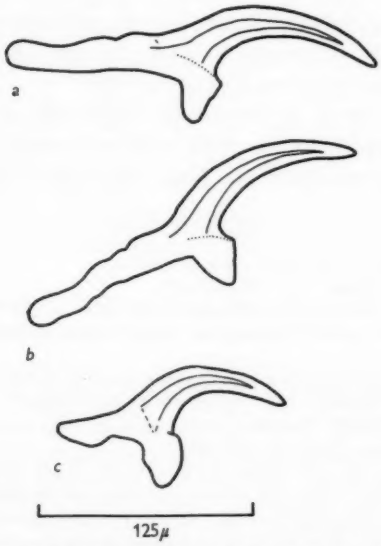

Fig. 5. *Taenia ingwei* Ortlepp, 1938. *a*, *b*, Rostellar hooks, anterior row; *c*, rostellar hook, posterior row.

Taenia ingwei Ortlepp, 1938 (Fig. 5)

Several fragmented worms, identified as the above species, were recovered from the intestine of a leopard, *Panthera pardus*, at Wankie. The scolex, 0·91–1·1 mm. in diameter, bears four rounded suckers which measure 0·27–0·28 mm. in diameter. There are 36–40 rostellar hooks, which are arranged in a double row. The hooks of the first row measure 195–220 μ long, and those of the second row 123–135 μ long. The cirrus sac measures 0·44–0·45 mm. long by 0·10 mm. wide. There are approximately 400 testes in each segment. The testes measure 0·08–0·09 mm. in diameter. The uterus has 5–10 primary branches on each side which may be subdivided. The anterior branches may form a multilobed sac at the anterior end of the segment. The eggs are slightly oval and measure 35–40 μ long by 33–35 μ wide.

Discussion

The above brief re-description is given because of differences between the present material and Ortlepp's original description. Ortlepp (1938), in describing *T. ingwei*, records that there are 32–34 hooks, of which the largest were 197–202 μ long, and the smaller 148–151 μ long. The cirrus sac was stated to have a maximum length of 0·435 mm. and is 0·087 mm. wide. In the number of testes, and uterine branches and the size of the eggs, the present specimens are similar to the type material described by Ortlepp.

SUMMARY

Three new species of cestodes are described. They are: *Joyeuxiella paucitestis* sp.nov. from the Rusty Spotted Genet, *Genetta rubiginosa*; *Diplopylidium paurodex* sp.nov. from the Rusty Spotted Genet, *Genetta rubiginosa*; *Taenia crocutae* sp.nov. from the Spotted Hyaena, *Crocuta crocuta*.

Re-descriptions are given of *Joyeuxiella fuhrmanni* (Baer, 1924), Fuhrmann, 1935, and *Taenia ingwei* Ortlepp, 1938, with particular reference to differences between the original descriptions and those based upon the present material.

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Studies on ovarian development in tsetse flies (*Glossina*, Diptera)

By D. S. SAUNDERS
Department of Zoology, University of Edinburgh

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I. INTRODUCTION

The female tsetse fly gives birth to a single 3rd-instar larva at about 10-day intervals throughout its reproductive life. This larva has a very short independent existence and burrows into the ground to pupate within a few minutes of birth.

In the female genital system there are several morphological features which are associated with this viviparous habit. The most apparent of these are the enlargement of the common oviduct to form the uterus, which contains the larva during the gestation period; the modification of the accessory glands to produce a secretion (the 'milk'), which nourishes the developing larva; and the great reduction in the size of the ovaries, a feature associated with the production of a very small number of eggs.

Each ovary of a tsetse fly has been shown (Saunders, 1960*a*, *b*) to consist of two polytrophic ovarioles, each of which, in a normal female, contains a single egg follicle. In the newly emerged fly (*G. morsitans*) the four ovarioles are at different stages of development, and the right ovary is more advanced than the left. The most advanced ovariole in the right ovary contains an egg follicle 0·4–0·5 mm. in length, a third of which is ovum and yolk; the second ovariole in this ovary shows the follicle just descending from the germarium. The most advanced ovariole in the left ovary contains a follicle 0·2—0·25 mm. in length, and shows the first signs of yolk deposition around the nucleus of the ovum. The smaller ovariole in the left ovary consists of a germarium and follicular tube only and shows no sign of follicular development. The first follicle to develop is the most advanced one in the right ovary. Only one egg is produced at a time, and a sequence of egg maturation and ovulation occurs so that the first and third eggs are produced from the

right ovary and the second and fourth eggs from the left. Subsequent eggs are produced alternately from right and left ovaries in a continuation of the same sequence.

This paper describes the development of the egg follicles in the pupa and the specific differences between the ovaries of five species of tsetse flies. It also considers the reasons for differences in the size of the egg follicles in newly emerged flies, and the effect of the bloodmeal on the development of the ovaries of *G. morsitans*.

II. MATERIALS AND METHODS

Pupae of several species of tsetse flies were obtained from various regions of Africa and sent to Edinburgh by air (see text for details). Most of the work was done with *G. morsitans*, pupae of which were received as a regular weekly supply from Singida, Tanganyika.

The flies were allowed to emerge in the laboratory either at normal room temperature (about 18° C.) or in an insectary at about 27° C. For experimental purposes many flies were allowed to emerge at 25° C. The newly emerged flies were kept singly in 3 × 1 in. glass tubes, each fitted with a nylon net top through which the fly fed, and a small circle of blotting-paper on the floor of the tube to absorb the liquid faeces. If not dissected immediately they were maintained by blood-feeding on the human hand. Most of these flies were incubated at 25° C. and at 60% relative humidity, and in many of the experiments the flies were weighed before and after feeding so that the weight of the bloodmeal could be determined. Female flies were mated with older, virgin males in the tubes in which they were kept.

The degree of development of the ovaries was determined by dissecting the flies in 0·9% saline on a microscope slide, and measuring the lengths of the egg follicles with a calibrated eyepiece micrometer. Permanent preparations of the follicles were made by draining the slides of the excess saline and mounting the follicles in polyvinyl-lactophenol in which was dissolved a small amount of lignin pink. This mountant stained and cleared the ovarioles in one operation and avoided the shrinkage associated with dehydration.

The convention adopted in this paper for naming the four ovarioles and the follicles which they contain, is as follows: the most advanced ovariole in the right ovary and the one which produces the first egg is called ovariole (and follicle) A, and the second ovariole (and follicle) in the right ovary is called B. The most advanced ovariole in the left ovary, producing the second egg in the sequence, is called C, and the second ovariole in the left ovary is called D. The second follicles produced in each ovariole after the ovulation of the first, are called A_2, B_2, C_2 and D_2. Thus the developmental cycle described in the introduction and in a previous paper (Saunders, 1960*b*) is A, C, B, D, A_2, C_2, B_2, etc.

One of the difficulties encountered during the present investigation was the great variation in physiology between individual flies. This variation is due to the fact that the flies used in these experiments emerged from pupae collected in the field. These pupae are produced by wild females, each exposed to different climatic

conditions, and deposited in different pupal sites, each subjected to widely differing temperatures, humidities and degrees of radiation. In addition to these sources of variation, the collected pupae are all of different ages and thus affected to a different degree by the adverse conditions met with during collection and despatch by air to Edinburgh. Thus the flies emerging in the laboratory from these pupae vary greatly in size, amount of fat, ability to take and digest the bloodmeal, size of the follicles, and in general vigour. Some of this variation is shown in the results given in this paper.

These difficulties could be overcome, to some extent, by using flies from a laboratory-bred colony of tsetse flies kept under constant or known conditions. Several species of *Glossina*—notably *G. palpalis* (W.A.I.T.R. reports, 1953–57) and less successfully, *G. morsitans* and *G. austeni* (Foster, 1957)—have been reared in laboratory culture, but these cultures are not fully self-supporting and impossible to maintain without adequate facilities. Unfortunately these were not available during the present investigation and the experiments had to be performed on the progeny of wild flies.

Throughout this work temperatures were controlled in incubators, and relative humidities were maintained in kilner jars over sulphuric acid and water mixtures (Solomon, 1951). Variable temperatures, such as in the insectary, were recorded by a mercury-in-steel thermohygrograph.

III. THE DEVELOPMENT OF THE OVARIES IN *GLOSSINA MORSITANS* PUPAE

The development of the egg follicles in adult *G. morsitans*, given ample opportunity to feed and kept at 26–27° C., has been described earlier (Saunders, 1960*b*), and the appearance of the ovaries in the newly emerged fly is described in the introduction. In order to trace the development of the egg follicles back to their first appearance in the ovary, the development of the follicles in the pupa and pharate adult is now described.

Because the *G. morsitans* pupae were collected in the field they were of variable and unknown age. For this reason they were kept at 25° C. and aged during dissection by the degree of pigmentation of the eyes, bristles and integument, a method described by Bursell (1959*a*). The ovaries were dissected in saline and the lengths of the egg follicles measured by means of a calibrated eyepiece micrometer.

The ovaries of an 8-day-old puparium (i.e. a puparium containing a newly formed pupa which is without pigmentation) each contain two ovarioles which consist of a germarium and follicular tube only. Egg follicles are first recognizable in 12-day-old puparia (containing a pupa with yellow eyes); these puparia show the first signs of the descent of follicles A and C from their germaria. Follicle A descends a short time before C and is consistently more advanced than the latter, this difference in size increasing as the pupa develops (Table 1). Just before the adult fly emerges from the puparium the follicle in ovariole B descends from the germarium; follicle D descends on the fourth day after emergence.

Yolk begins to be deposited in the follicles of *G. morsitans* when they reach a length of about 0·20–0·22 mm. Follicle A reaches this length in the 21-day-old

puparium, about 7 days before emergence, and follicle C reaches this length in the 24-day-old puparium, about 4 days before emergence. Yolk deposition has therefore started before the fly emerges, and in the newly emerged fly at least one-third of follicle A consists of the yolk-filled oocyte.

Table 1. *The lengths of the egg follicles in pupae of* Glossina morsitans *maintained at* 25° C., *showing that the sequence of egg development is established during pupal development*

Age of puparia (days)*	Description of pupa within puparium	No. of puparia dissected	Length of egg follicles (mm.)				Difference between A and C (mm.)
			A	B	C	D	
8	Newly formed pupa with no pigmentation	10	No signs of follicles				—
12	Eyes only pigmented: yellow	7	f.s.	—	f.s.	—	—
16	Eyes reddish brown	5	0·156	—	0·116	—	0·040
21	Eyes pigmented, bristles black	10	0·230	—	0·186	—	0·044
24	Integument pigmented; skin moist	12	0·324	—	0·239	—	0·085
26	Integument pigmented, skin dry (pharate adult)	8	0·458	—	0·248	—	0·210
	Teneral fly	14	0·538	f.s.	0·258	—	0·280

* Aged by Bursell's (1959*a*) method.
f.s. = first sign of descent of follicle from the germarium.

IV. THE OVARIES OF NEWLY EMERGED FLIES

The females of five species of *Glossina* have been dissected soon after emergence. In all of them each ovary contained two polytrophic ovarioles of the same general pattern as that described for *G. morsitans*. The specific differences in the ovaries of these five species are described below.

(1) *Glossina morsitans* Westw. (Fig. 1I)

Over 300 flies of this species have been dissected at various periods of the ovulation cycle (Saunders, 1960*b*) and in all of them the first egg was produced from the right ovary and the pattern of the ovulation cycle never varied. In newly emerged flies the right ovary is always more advanced than the left. Table 2 shows the mean lengths of the egg follicles of ten females which emerged from a batch of pupae collected during July 1960, and the variation in the size of the follicles which occurs between individuals from the same batch.

(2) *G. pallidipes* Aust. (Fig. 1II)

A batch of pupae of this species was obtained in January 1960, from Kabete, Kenya. They were kept in the laboratory under the same conditions as the *G. morsitans* and nine females were obtained. As in *G. morsitans*, each ovary consists of two ovarioles and the right ovary is more advanced than the left at emergence.

Table 2. *The size of the egg follicles in newly emerged tsetse flies*

Species of *Glossina*	No. of flies	Size (R.D.W.)	Length of egg follicles in mm. A	B	C	D
G. austeni	2	3·2	0·370 (0·37, 0·37)	—	0·190 (0·18–0·20)	—
G. morsitans	10	6·3	0·493 (0·37–0·54)	f.s.	0·254 (0·24–0·29)	—
G. pallidipes	9	6·7	0·534 (0·34–0·69)	f.s.	0·233 (0·15–0·31)	—
G. brevipalpis	6	11·8	0·697 (0·58–0·84)	0·174 (f.s. −0·19)	0·345 (0·27–0·60)	0·174 (0–0·31)

R.D.W., Residual dry weight.

f.s. = first sign of egg follicle descending from the germarium (the figures in brackets indicate the variation in follicle size).

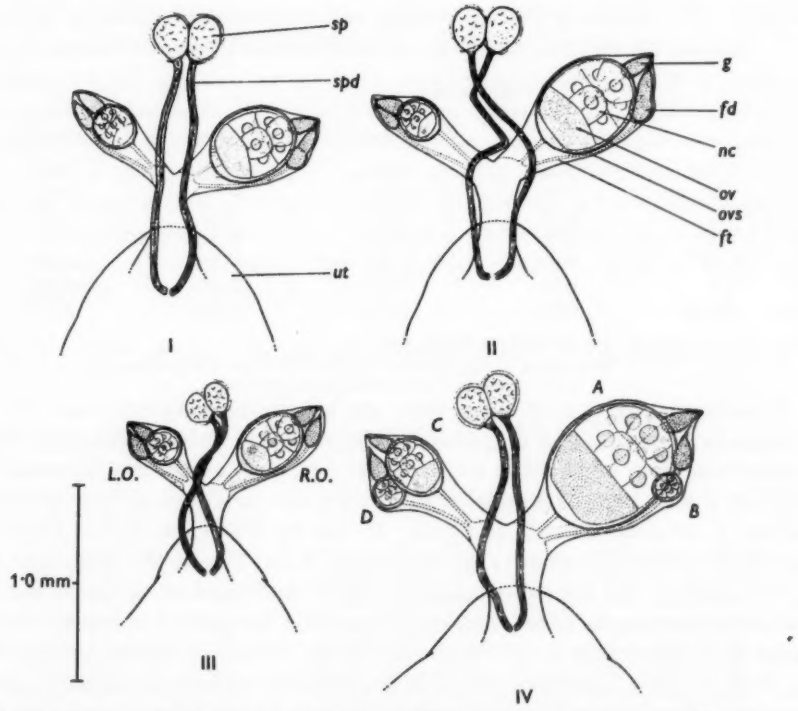

Fig. 1. The ovaries of newly emerged tsetse flies. I: *Glossina morsitans* Westw.; II: *G. pallidipes* Aust.; III: *G. austeni* Newst.; IV: *G. brevipalpis* Newst. *L.O.* Left ovary; *R.O.* right ovary; A, B, C, D, ovarioles A, B, C, D; *fd* = follicle descending from germarium; *ft* = follicular tube; *g* = germarium; *nc* = nurse cells; *ov* = ovum; *ovs* = ovarian sheath; *sp* = spermatheca; *spd* = duct of spermatheca; *ut* = uterus.

The lengths of the egg follicles were measured and are shown in Table 2. Although only a small sample of flies have been examined, these figures show that there is considerable variation in the size of follicles A and C at emergence; this variation is greater than that in a batch of *G. morsitans* of similar size. The mean lengths of the follicles show that the ovaries of *G. pallidipes* are slightly more advanced than those of *G. morsitans* when the fly emerges. About a third of follicle A consists of yolk, follicle B is just descending from the germarium and ovariole D has not yet developed its follicle. No flies were fed or kept longer than one day.

(3) *G. brevipalpis* Newst. (Fig. 1IV)

Several batches of *G. brevipalpis* were obtained from Kabete, Kenya, and also, from Zululand. In this species, each ovary also contains two ovarioles and the right ovary is more advanced than the left at emergence. Measurements of the follicles are shown in Tables 2 and 3.

Table 3. *The lengths of the egg follicles in newly emerged* Glossina brevipalpis *showing the variation in follicle size which occurs between individual flies*

	Right ovary		Left ovary	
	A (mm.)	B (mm.)	C (mm.)	D (mm.)
1	0·84	0·19	0·27	0·19
2	0·81	0·19	0·33	0·31
3	0·75	f.s.	0·30	—
4	0·60	0·17	0·60	0·12
5	0·60	0·19	0·30	0·12
6	0·58	0·13	0·27	0·13
Means	0·697	0·174	0·345	0·174

Each line concerns a single fly.
f.s. = first signs of egg follicle descending from the germarium.

Follicles A and C in *G. brevipalpis* are larger at emergence than the same follicles in *G. morsitans* or *G. pallidipes*. The figures in Table 3 also show that the ovaries are more *advanced* in *G. brevipalpis*; in most of the flies dissected both follicles B and D have descended from their germaria and at least one-half of follicle A consists of ovum and yolk. In one fly (2) follicle D was larger than follicle B and in two others (1 and 6) follicles B and D were the same size. In fly no. 4 follicle C was the same length as follicle A. Therefore, although the mean values for the lengths of the follicles indicate that the pattern of ovarian development in *G. brevipalpis* is similar to that in the other two species described (e.g. A, C, B, D, etc), there appears to be considerable individual variation and the sequence of egg maturation and ovulation may be erratic. Thus it is possible that a few females may produce the first egg from the left ovary, or two consecutive eggs from the same ovary. The ovulation cycle in this species, however, could not be investigated because of the small number of flies available.

(4) *G. austeni* Newst. (Fig. 1III)

Several batches of *G. austeni* pupae were received from Zululand but most of them were dead on arrival, probably because of the long journey from the collecting

grounds to the point of despatch (Onderstepoort, Transvaal.) Two female flies, however, emerged from these pupae. In the first fly, follicle A was 0·37 mm. and follicle C 0·18 mm. in length, and in the second fly follicle A was 0·37 mm. and follicle C 0·20 mm. in length. In both flies, neither follicle B nor D had descended from their germaria, and about one-quarter of follicle A consisted of ovum and yolk. Therefore, although no conclusions should be drawn from only two specimens, it appears that the ovaries of this small species are not so far advanced as those of the other, larger species dissected.

(5) *G. palpalis* (R.-D.)

A few pupae of *G. palpalis* were kindly supplied by Mr W. A. Page who obtained them from Kaduna, Northern Nigeria. A number of females were dissected but no measurements of the egg follicles were made. The appearance of the ovaries of this species at emergence, however, is very similar to that of *G. morsitans*.

V. VARIATIONS IN THE SIZE OF THE FOLLICLES OF *GLOSSINA MORSITANS* AT EMERGENCE

Although there has been no variation observed in the sequence of egg development and ovulation in *G. morsitans*, it has been shown (Table 2) that there is considerable variation in the size of the follicles between individual newly emerged flies. Table 4 shows that there is also a variation in follicle size between flies emerging from batches of pupae collected at different times of the year. For instance, flies emerging from pupae collected in October 1959 had smaller follicles (A = 0·41 mm., C = 0·21 mm.) than those from pupae collected in March 1960 (A = 0·54 mm., C = 0·26 mm.).

Table 4. *The lengths of the egg follicles of newly emerged* Glossina morsitans, *showing a seasonal variation in size*

Month in which pupae were collected	No. of flies dissected	Length of the egg follicles in mm.			
		Right ovary		Left ovary	
		A	B	C	D
Oct. 1959	5	0·410 (0·38–0·45)	f.s.	0·210 (0·20–0·23)	—
Jan. 1960	5	0·486 (0·43–0·51)	f.s.	0·226 (0·21–0·27)	—
Mar. 1960	14	0·538 (0·39–0·70)	f.s.	0·258 (0·24–0·27)	—
May 1960	19	0·471 (0·38–0·54)	f.s.	0·238 (0·21–0·27)	—
July 1960	10	0·493 (0·37–0·54)	f.s.	0·254 (0·24–0·29)	—
Aug. 1960	10	0·463 (0·34–0·53)	f.s.	0·221 (0·17–0·25)	—
Sept. 1960	10	0·451 (0·42–0·49)	f.s.	0·225 (0·20–0·25)	—
Oct. 1960	11	0·433 (0·37–0·46)	f.s.	0·220 (0·19–0·23)	—

Before yolk deposition begins, an egg follicle of *G. morsitans* grows to a length of about 0·20–0·22 mm. Increase in size after this is due to the increase in the number of nurse cells from 7 to 14 (Saunders, 1960*b*) and to deposition of yolk in the oocyte. Follicle A at emergence contains 14 nurse cells and variation in the size of this follicle is due to differences in the amount of yolk laid down. The size of the follicle, therefore, is an indication of its state of development. From this it

follows that some flies have more highly developed follicles at emergence, these differences occurring between individual flies and between flies at different times of the year.

It has been shown (Tables 2 and 3, and Fig. 1) that *G. brevipalpis*, a large fly, has, at emergence, larger follicles with more yolk than *G. morsitans* or *G. pallidipes*, and *G. austeni*, which is a small fly, has smaller follicles with much less yolk. This suggests that the size of the follicles at emergence is a function of the size of the fly. This was tested in an experiment in which an attempt was made to correlate the size of the follicles of newly emerged *G. morsitans* with the body size of the fly.

The measurement of size in tsetse flies has been based on three criteria: residual (fat free) dry weight, the length of the middle part of the fourth longitudinal wing vein (Jackson, 1946), and a measure of thoracic surface 'area' (Bursell, 1960*a*). Residual dry weight (R.D.W.), which is independent of any water loss or the amount of fat in the fat-body, is considered (Bursell, 1960*a*) to be the most accurate method of determining size for extruded larvae, pupae or teneral flies. In non-teneral flies, however, the presence of a bloodmeal in the gut or, as in the case of a pregnant female, a larvae in the uterus, renders R.D.W. useless as a measure of size and the two latter measures of size are more accurate. In teneral *G. morsitans*, Bursell showed that, for both sexes, the 'area' of the thoracic surface (the product of the distance between the points of insertion of the largest of the lateral pronotal spines and of the distance between the base of the scutellar spines and the mesonotal suture) was highly correlated with R.D.W. and hence an accurate measure of size. The length of the middle part of the fourth wing vein was not so well correlated with R.D.W. and hence was a poorer measure of size. In this investigation only newly emerged flies were used; therefore, size was measured by means of R.D.W. determinations, and the other two measures were used only as a comparison.

Method

Female *G. morsitans*, less than 2 hr. old, were killed in ether vapour and weighed on a torsion balance. The length of the middle part of the fourth wing vein and the thoracic surface 'area' were then measured in each fly by means of a calibrated eyepiece micrometer. A small cut was made around the terminal abdominal segment and the ovaries drawn out, together with a short piece of hind gut, in such a manner that the fat-body was left in the abdomen. The lengths of follicle A (right ovary) and follicle C (left ovary) were then measured and all pieces of tissue replaced on or in the abdomens of the flies. This procedure caused a certain degree of error in the experiment because small pieces of tissue were inevitably lost and small amounts of saline added to the flies during manipulation, but these sources of error were unfortunately unavoidable.

In the first experiment of this type the flies were frozen and then dried in a vacuum desiccator (three times) to constant weight in the presence of P_2O_5 and concentrated H_2SO_4. In the second experiment the flies were dried in a vacuum desiccator in an oven at 83° C. There was little difference evident in the results gained from these two methods, although it is felt that the first has an advantage over the second in that possible changes in weight due to oxidation or losses of more

volatile solids at high temperature are probably avoided. After being dried, the flies were transferred to a vessel containing anhydrous $CaCl_2$ and only removed immediately before weighing on the torsion balance; in this way the absorption of water from the atmosphere was kept to a minimum. The flies were then placed individually in tubes of cold chloroform to extract the fat (presumably mainly triglycerides from the fat-body); extraction in chloroform was repeated several times and the flies finally dried twice and weighed. This procedure gave, apart from a measure of R.D.W., an estimation of water and fat content of each fly.

Results

The results of the first experiment using nineteen flies which emerged from pupae newly received from Singida and collected during May 1960 are shown in Table 5. The residual dry weights of these flies varied between 4·7 and 7·0 mg. (mean R.D.W. = 6·26 mg.), follicle A varied between 0·40 and 0·53 mm. (mean length = 0·471 mm.) and follicle C varied between 0·21 and 0·27 mm. (mean length = 0·238 mm.). Table 5 also shows the fat content of each fly (mean = 2·06 mg., 24·76 % of the dry weight). Water content varied between 23·7 mg. (79·26 % of the fat-free weight) and 15·4 mg. (76·62 %) (mean = 20·06 mg., 76·07 %).

Attempts were made to correlated the three measures of size (R.D.W., the length of the middle part of the fourth wing vein, and the thoracic surface 'area') with the lengths of both egg follicles. It was found that R.D.W. was positively correlated with the length of follicle A ($r = +0{\cdot}5695$, $P = <0{\cdot}02$), thoracic surface 'area' was also correlated ($r = 0{\cdot}4417$, $P = 0{\cdot}05$), although to a lesser extent, but the middle part of the fourth wing vein was not (Table 5). Follicle C was not so well correlated with the size of the fly, presumably because, at the time of emergence, the nurse cells of this follicle are undergoing multiplication in number from 7 to 14. It was also found that the thoracic surface 'area' was well correlated with R.D.W. ($r = +0{\cdot}7846$, $P = <0{\cdot}001$). This result confirms that of Bursell (1960*a*).

The second experiment used nineteen flies which emerged from pupae collected in August 1960. These flies were smaller than the first group (R.D.W. = 4·8–6·9 mg., mean = 5·76 mg.) and follicle A was smaller (0·41–0·53 mm., mean length = 0·461 mm.). These flies also contained less fat (mean fat content = 1·59 mg.). Residual dry weight was positively correlated with the length of follicle A, although less significantly so than in the first experiment ($r = +0{\cdot}4294$). The thoracic surface 'area' was also correlated ($r = +0{\cdot}4599$), but the r value for the middle part of the fourth wing vein and the length of follicle A did not differ significantly from zero ($r = +0{\cdot}0039$). Correlations of the latter two measures of size with R.D.W. gave $r = +0{\cdot}8331$ and $r = +0{\cdot}2828$ respectively. If the results for the two experiments are combined a correlation between the size of follicle *A* and R.D.W. of $r = +0{\cdot}4997$, $P = <0{\cdot}01$ is obtained.

The figures in table 5 also show that the fat content (weight of chloroform-extractable material) of the flies is correlated with R.D.W. ($r = +0{\cdot}6818$, $P = <0{\cdot}01$), a result which is in agreement with that of Jack (1941) and Bursell (1960*b*). These figures also suggest that large follicles are associated with a large amount of fat in the fat-body, and, although in this investigation a significant correlation

could not be found between these two variables ($r = +0{\cdot}0801$), it seems probable that the state of the reserves in the fat-body would affect the amount of yolk deposited in the oocyte before emergence.

Table 5. *The lengths of the egg follicles in newly emerged* Glossina morsitans, *showing that they are correlated with the size of the fly*

Size of the fly						
Fresh weight (mg.)	Length of middle part of 4th wing vein (mm.)	Thoracic surface 'area' (arb. units)	Dry weight (mg.)	Residual dry weight (mg.)	Weight of fat (mg.)	Length of follicle A (mm.)
30·8	1·806	9·053	9·3	7·0	2·3	0·53
31·8	1·849	9·313	9·3	7·0	2·3	0·53
32·1	1·720	8·542	9·5	7·0	2·5	0·51
29·8	1·655	8·165	9·0	6·9	2·1	0·50
30·5	1·741	7·921	9·3	6·8	2·5	0·46
29·8	1·763	8·520	9·0	6·7	2·3	0·48
29·8	1·784	8·796	8·6	6·6	2·0	0·51
25·2	1·763	7·921	8·2	6·3	1·9	0·53
27·9	1·827	8·520	8·3	6·3	2·0	0·40
31·7	1·698	9·313	8·0	6·2	1·8	0·46
29·8	1·784	8·542	9·0	6·2	2·8	0·43
29·0	1·827	8·514	8·7	6·2	2·5	0·38
27·8	1·763	8·025	8·3	6·2	2·1	0·51
27·6	1·784	8·548	7·7	6·0	1·7	0·48
24·9	1·784	7·795	7·5	6·0	1·5	0·46
26·1	1·720	7·921	7·7	5·7	2·0	0·46
26·7	1·784	8·413	7·2	5·6	1·6	0·46
26·5	1·763	8·047	7·5	5·5	2·0	0·43
21·3	1·591	6·730	5·9	4·7	1·2	0·42

Correlations (r values)

	Length of follicle A	Thoracic surface 'area'	Length of middle part of 4th wing vein	Weight of fat
Residual dry weight	+0·5695 ($P = <0{\cdot}02$)	+0·7846 ($P = <0{\cdot}001$)	+0·3846 (not sig.)	+0·6818 ($P = <0{\cdot}01$)
Thoracic surface 'area'	+0·4417 ($P = 0{\cdot}05$)	—	—	—
Length of middle part of 4th wing vein	+0·0499 (not sig.)	—	—	—
Weight of fat	+0·0801 (not sig.)	—	—	—

In Fig. 2 data for the length of follicle A in *G. austeni* and *G. brevipalpis* have been added to the graph (but not included in the calculation of the regression). The size of follicle A in *G. austeni* is based on two females whose mean R.D.W. was determined as 3·2 mg. The follicle length for *G. brevipalpis* is the mean of six females; the R.D.W. of this species (11·8 mg.) is taken from Bursell (1959*b*). It is of interest that the results for these two species fall close to the line calculated for *G. morsitans*.

This indicates that the same sort of relationship between the size of the fly and the size of the follicles at emergence holds true between species as well as between individuals of the same species.

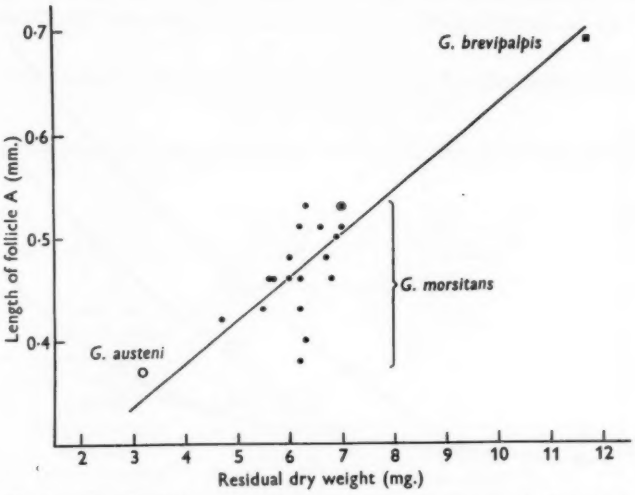

Fig. 2. The length of follicle A (right ovary) in newly emerged *Glossina morsitans* is correlated with the size (residual dry weight) of the fly. Data for *G. austeni* and *G. brevipalpis* (mean values) have been added to show that they fall close to the line calculated for *G. morsitans*. The regression calculated for *G. morsitans* is $y = 0{\cdot}0422x + 0{\cdot}207$ ($r = +0{\cdot}5695$, $P = <0{\cdot}02$).

VI. THE EFFECT OF THE BLOODMEAL UPON OVARIAN DEVELOPMENT IN *GLOSSINA MORSITANS*

Some yolk deposition has been shown to occur in follicle A (right ovary) and follicle C (left ovary) before emergence. In flies given ample opportunity to feed this growth continues after emergence and at 26–27° C. follicle A reaches maturity about 8–9 days after emergence, and follicle C reaches maturity about 20 days after emergence (Saunders, 1960*b*). Very little work has been done on the factors affecting this growth. In this investigation the effect of the bloodmeal was studied. In all of the experiments attention was principally given to the growth of follicle A which develops most rapidly in the first few days after emergence.

(1) *Growth of the egg follicles in unfed flies*

In the laboratory at 25° C. and 60% relative humidity most unfed female *G. morsitans* survive for 4 days. A few flies live longer than 4 days, and in the present investigation one female died on the 9th day after emergence. This, however, is unusual, and for this reason the growth of the egg follicles was measured only during the first 4 days of life.

The deposition of yolk in the oocyte which begins before the fly emerges from the puparium continues in the unfed fly (Table 6 and Fig. 3). In the batch of flies used for this experiment, follicle A increased in length from 0·486 mm. at emergence to

0·566 mm. on the fourth day, and follicle C grew from 0·226 to 0·251 mm. The increases of both follicles are significant (A, $t = 2{\cdot}002$, $P = <0{\cdot}05$; C, $t = 2{\cdot}128$, $P = <0{\cdot}05$). In a later batch of flies, follicles A and C increased from 0·464 to 0·650 mm. and from 0·256 to 0·271 mm. respectively.

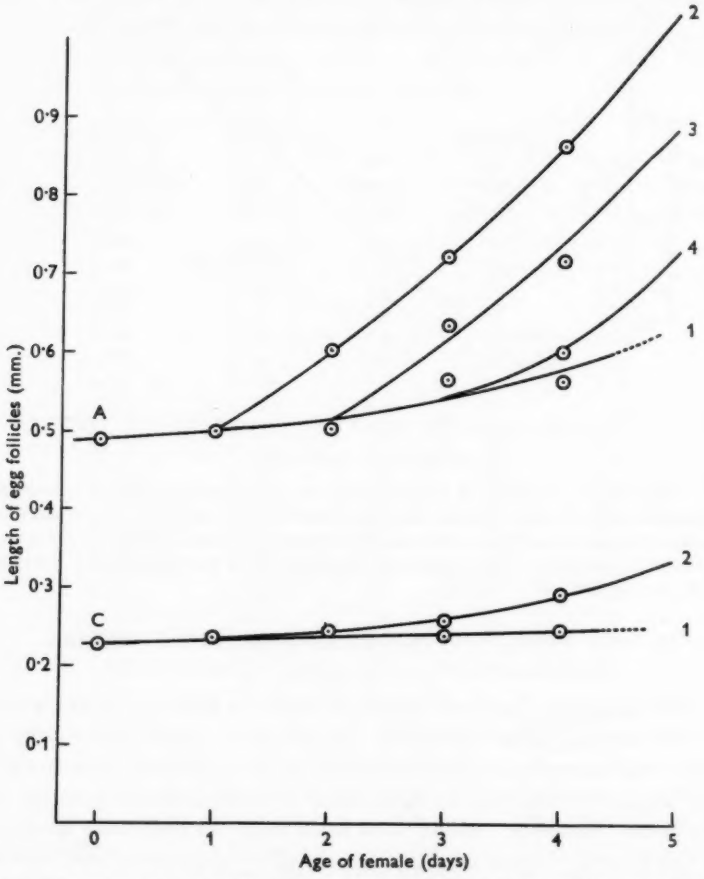

Fig. 3. The growth of follicles A (right ovary) and C (left ovary) in (1) unfed flies, (2) flies fed once 24 hr. after emergence, (3) flies fed once 2 days after emergence, (4) flies fed once 3 days after emergence.

(2) *Growth of the egg follicles in fed flies*

Flies were fed on the 1st, 2nd or 3rd days after emergence, and then kept at 25° C. and 60 % relative humidity until dissection. These flies were weighed just before and just after feeding, so that the weight of the bloodmeal, which varied between 10 and 47 mg. at the first feed, could be expressed as a percentage of the fresh body weight of the fly. In this way an estimation of the relative size of the bloodmeal could be found. The results of these weighings in a total of 124 flies (Table 7) show that the mean weight of blood taken at the first feed increases as

the flies get older and more hungry. This result confirms the observations of Jack (1939) on the size of the bloodmeal taken by *G. morsitans* in Southern Rhodesia.

The growth of the follicles which occurs in unfed flies is accelerated after the fly takes a bloodmeal (Table 6 and Fig. 3). Twenty-four hours after feeding the bloodmeal is practically digested and the fat body has increased in size. In flies fed

Table 6. *The growth of the egg follicles in unfed and fed* Glossina morsitans *at* 25° C. *and* 60% (*m.m.*) R.H.

(The fed flies received a single bloodmeal on the 1st, 2nd or 3rd day after emergence.)

Age of fly (days)	Unfed	Fed at 24 hrs.	Fed at 48 hrs.	Fed at 3 days
		(*a*) Follicle A, right ovary		
0	0·486 (5) S.E. = 0·01314	—	—	—
1	0·500 (5) S.E. = 0·01414	—	—	—
2	0·502 (3) S.E. = 0·02216	0·604 (4) S.E. = 0·00817	—	—
3	0·586 (5) S.E. = 0·01947	0·723 (4) S.E. = 0·00818	0·636 (9) S.E. = 0·01917	—
4	0·566 (12) S.E. = 0·02132	0·866 (10) S.E. = 0·03852	0·720 (3) S.E. = 0·01269	0·602 (8) S.E. = 0·03422
6	—	1·088 (18) S.E. = 0·06030	1·097 (13) S.E. = 0·05436	1·023 (11) S.E. = 0·04449
8	—	1·232 (15) S.E. = 0·04707	1·223 (11) S.E. = 0·03392	1·122 (21) S.E. = 0·04123
		(*b*) Follicle C, left ovary		
0	0·226 (5) S.E. = 0·01004	—	—	—
1	0·239 (5) S.E. = 0·00443	—	—	—
2	0·246 (3) S.E. = 0·01440	0·247 (4) S.E. = 0·00376	—	—
3	0·244 (5) S.E. = 0·00727	0·262 (2) S.E. = 0·00376	0·242 (9) S.E. = 0·00491	—
4	0·251 (12) S.E. = 0·00604	0·297 (10) S.E. = 0·00697	0·255 (3) S.E. = 0·00707	0·252 (8) S.E. = 0·00727
6	—	0·323 (18) S.E. = 0·00832	0·361 (13) S.E. = 0·01371	0·354 (11) S.E. = 0·00921
8	—	0·438 (15) S.E. = 0·01419	0·407 (11) S.E. = 0·00717	0·408 (21) S.E. = 0·01262

Figures in parentheses indicate the number of flies dissected.

24 hr. after emergence, the follicles are larger 1 day after feeding than the corresponding follicles in unfed flies. This difference increases daily and on the 4th day (3rd day after feeding) follicle A was 0·866 mm. in fed flies compared with only 0·566 mm. in unfed flies, and follicle C was 0·297 mm. in length compared with 0·251 mm. The increase for follicle A is significant ($t = 2{\cdot}411$, $P = 0{\cdot}02$).

After the 4th day the follicles continue their development and the fat-body becomes gradually depleted. On the 8th day (7 days after feeding) the mean

length of follicle A was about 1·2 mm. and C about 0·4 mm. Although some flies develop follicles as great as 1·4 mm. in length after 8 days, no mature eggs were observed, and after 10 days flies were dying from starvation with a very depleted fat-body and an egg in the right ovary which was undergoing degeneration. A single bloodmeal (even if over 200 % of the fresh body weight of the fly), therefore, is insufficient for the maturation of the first egg, a situation unlike most of the blood-sucking Nematocera and Brachycera which show gonotrophic concordance. Flies fed twice (once 24 hr. after emergence and again on the 5th day), however, were able, in some instances, to develop a mature egg in the right ovary.

Table 7. *The size of the first bloodmeal taken by female* G. morsitans, *showing that larger bloodmeals are taken by older flies*

Age of flies when fed (days)	No. of flies	Mean weight of flies (mg.)	Mean weight of blood (mg.)	Bloodmeal as percentage of fresh weight of fly
1	68	24·5 (17–33)	20·2 (10–33)	82·45 (37·0–129·4)
2	24	23·4 (19–29)	31·2 (17–40)	133·33 (62·9–200·0)
3	32	22·6 (18–30)	33·1 (14–47)	146·46 (50·0–233·6)

The figures in parentheses indicate the variation.

Some of the flies were fed on the 2nd or 3rd day after emergence instead of on the first. The effect of this delay in the bloodmeal was to delay the acceleration in growth of the follicles which occurs in fed flies, on the 4th day after emergence the follicles of a fly fed on the 2nd or 3rd day being smaller than those fed on the 1st day (Fig. 3, Table 6). After about the 6th day, however, individual variations in follicle size become too great and there is no significant difference between the sizes of follicles of flies fed on different days.

Each fly used in the experiments described above was weighed before and after feeding so that both the weight of the fly and of the bloodmeal were known. There was no correlation, however, between the size of the bloodmeal (expressed as a percentage of the fresh body weight of the fly, or as a function of the size of the fly as determined by measurements of the thoracic surface 'area') and the amount of growth occurring in the egg follicles. For instance, a fly taking 15 mg. of blood (57·7 % of its weight) at its first feed developed larger follicles than a fly taking 31 mg. of blood (110·7 %). In another experiment in which flies were fed on the third day after emergence, the largest bloodmeal was 42 mg. (233·6 % of the fly's body weight) and the smallest was 18 mg. (75·0 %). After 8 days, follicle A in the first fly was only 0·91 mm. but the same follicle in the second fly was 1·28 mm. This lack of correlation was confirmed in an experiment in which flies were interrupted during the bloodmeal so that only a small amount of blood was ingested. Two sets of controls were used: fully fed flies and unfed flies. The results (Table 8) show that after 4 days follicles A and C in the unfed flies had grown to 0·650 and 0·271 mm.

respectively, and the same follicles in both sets of fed flies had grown much more, confirming the results shown earlier. There was no significant difference, however, between the size of the follicles in fully fed flies (A = 0·799 mm., C = 0·284 mm.) and those in the partially fed flies (A = 0·780 mm., C = 0·286 mm.) even though the mean size of the bloodmeal in the first group was 21·2 mg. (96·01 % of the body weight) and in the second group only 7·6 mg. (28·75 %). The smallest bloodmeal taken was 2·0 mg. (8·0 %); in this fly the lengths of the follicles were also very near the mean, follicle A being 0·75 mm. and follicle C 0·29 mm. Clearly, the size of the bloodmeal has no effect upon the amount of growth occurring in the egg follicles, and even the smallest bloodmeal is sufficient for normal development during the first few days.

Table 8. *The length of the egg follicles, in partially and fully fed* Glossina morsitans *after* 4 *days at* 25° C., *showing that the size of the bloodmeal has no effect upon the amount of growth occurring in the egg follicles*

	No. of flies	Mean weight bloodmeal (mg.)	Bloodmeal as percentage fresh weight	Length of egg follicles (mm.) A	Length of egg follicles (mm.) C
Unfed flies	8	—	—	0·650	0·271
				S.E. = 0·03491	S.E. = 0·01441
Partially fed flies	13	7·6	28·75	0·780	0·286
		S.E. = 0·8779		S.E. = 0·02058	S.E. = 0·00571
Fully fed flies	8	21·2	96·01	0·799	0·284
		S.E. = 1·4979		S.E. = 0·05272	S.E. = 0·00769

In a previous paper (Saunders, 1960*b*) figures were given which showed that, during the first pregnancy (9–21 days), the mean length of follicle C was significantly greater in pregnant females than in virgin females of the same age. It was suggested that this difference might be associated with the very large bloodmeals taken by pregnant flies. However, in the light of the present evidence that the size of the bloodmeal has no effect upon the amount of growth occurring in the egg follicles during the development of the first egg, this view may be untenable. On the other hand, it is possible that some other factor is at work in the pregnant female, or that repeated large bloodmeals may cause an increased development of the follicles.

VII. DISCUSSION

(1) *The type of ovary in tsetse flies*

Minchin (1905), Stuhlman (1907), Roubaud (1909) and all later authors (Buxton, 1955) considered that each ovary of *Glossina* consisted of a single ovariole containing four or five egg follicles. Hagen (1951), who studied the embryology and the ovaries of *G. tachinoides*, was also of this opinion; and the ovary of *Glossina* is often quoted as being an example of the extreme reduction in the number of ovarioles which can occur in insects (Wigglesworth, 1950). In this investigation, however, five species of tsetse fly have been shown to possess ovaries containing *two* polytrophic ovarioles each containing only a single egg follicle. The species examined

are representatives of all three 'groups' of the genus: *G. brevipalpis* is a member of the 'fusca group' (subgenus *Austenina*), *G. palpalis* belongs to the 'palpalis group' (subgenus *Nemorhina*), and *G. morsitans*, *G. pallidipes* and *G. austeni* belong to the 'morsitans group' (subgenus *Glossina*). In most respects the morphology of the 22 known species of *Glossina* is very consistent. There is no reason to suppose, therefore, that any other type of ovary will be found in the species of tsetse fly yet to be examined.

(2) *The size of the egg follicles at emergence*

It has been shown that the size of follicle A at emergence depends upon the size of the fly, large female *G. morsitans* having larger follicles with more yolk than small females. This relationship also exists between different species; *G. brevipalpis*, which is a very large fly (R.D.W. about 11–12 mg.), has much larger and more highly developed follicles at emergence than the smaller species *G. morsitans* and *G. pallidipes*. In *G. brevipalpis*, all four follicles have descended from their germaria, whereas in the latter two species only follicles A and C have completely descended and follicle B shows only the first signs of its descent. *G. austeni*, which is one of the smallest species of tsetse fly (R.D.W. about 3·2 mg.), has much smaller follicles with less yolk deposited before emergence, and no sign of follicle B.

Apart from a difference in size of the follicles between species and between flies of the same species, it has also been demonstrated that there is a variation in follicle size between flies emerging from pupae collected at different times of the year. This suggests that seasonal variations in climatic conditions affect the size of the follicles in newly emerged flies.

Buxton (1955) pointed out that the great variation in the size (weight) of newly emerged tsetse flies in the field is probably the result of the conditions acting upon the pupa or upon the female during pregnancy. In the laboratory, H. Mellanby (1937) showed that underfeeding of pregnant *G. palpalis* caused either abortion or the production of very small pupae. Buxton & Lewis (1934) showed that high temperature (30° C.) acting upon female *G. tachinoides* during the gestation period resulted in smaller pupae than those produced by females kept at low temperature (23° C.). At Gadau, Northern Nigeria, Buxton & Lewis demonstrated that the weights of both male and female *G. tachinoides* fell with increasing temperature during the hot months of February to May, and Jack (1939) showed a similar seasonal effect of temperature on the size of *G. morsitans* in Southern Rhodesia. For these reasons it seems likely that the differences in the size of the follicles of *G. morsitans* at emergence, which have been shown to be correlated with the size of the fly, are a reflexion of temperature effects acting upon the females during pregnancy.

Jack (1941) showed that larger *G. morsitans* pupae (and hence teneral adults) have proportionately more fat than smaller pupae, and that seasonal differences occur, flies produced during hot weather having smaller fat bodies than those produced during cooler weather. Bursell (1960*b*) also showed that fat consumption in *G. morsitans* pupae is greatly affected by the temperature. In pupae incubated at high temperature (above about 24° C.) the rate of fat consumption is greatly

increased without a correspondingly great reduction in the duration of the pupal period. At low temperature, the pupal period is greatly lengthened without a corresponding decrease in the rate of fat consumption. Therefore flies emerging from pupae exposed to either high or low temperatures contain less fat than those emerging from pupae incubated at the optimum temperature of about 22–24° C. These flies, therefore, will have less fat available, before the first bloodmeal, for deposition as lipid yolk in the oocytes. Protein reserves, to be utilized in the production of protein yolk are probably also affected by temperature in this way. For this reason it is probable that high or low temperature acting upon the pupa in the field may have some effect upon the state of development of the egg follicles in the teneral fly.

In the present investigation, the smallest follicles were recorded from flies which emerged from pupae collected during October, at the end of the long dry season. These pupae, therefore, and the generation of flies which produced them, would have been subjected to relatively high temperatures. The largest follicles were recorded from flies which emerged from pupae collected in March; these pupae and females which produced them probably lived under cooler conditions.

(3) *The effect of the bloodmeal upon ovarian development in* Glossina morsitans

Wigglesworth (1936) showed that the deposition of yolk in the oocytes of *Rhodnius prolixus* was controlled by a hormone secreted by the corpus allatum. Since then it has been shown in several other insects (see Wigglesworth, 1954) that egg development is under some sort of hormonal control, the hormone being secreted by the corpus allatum or by the neurosecretory cells of the brain. In many blood-sucking insects, such as *R. prolixus* (Wigglesworth, 1936), *Cimex lectularius* (K. Mellanby, 1939) and anautogenous mosquitoes (Detinova, 1945; Gillett, 1956, 1957; Clements, 1956), it has been shown that the egg follicles grow in the unfed female until they reach the point at which yolk deposition should commence. Further growth does not then occur until after the bloodmeal has been taken. Detinova (1953) showed that the growth of the ovaries of *Anopheles maculipennis* was only activated after a 'complete' bloodmeal; i.e. when the amount of blood ingested was equal to, or greater than, the body weight of the mosquito. After an incomplete bloodmeal the ovaries failed to develop. These results indicated that the stimulus for the release of the hormone from the corpus allatum was supplied by the pressure of a complete bloodmeal on the walls of the midgut or the body wall.

Experiments to demonstrate hormonal control of egg development in *Glossina* have not been attempted. If, however, yolk deposition in the follicles of tsetse flies *is* controlled by such a mechanism (and it seems not unlikely in view of the evidence from other Diptera, such as the mosquitoes, blowflies, (Day, 1943; Thomsen, 1952) and *Drosophila* (Vogt, 1953)), it differs in several important respects from the other examples of blood-sucking insects quoted above. In the five species of *Glossina* studied in this paper yolk deposition has already begun before the fly emerges from the puparium, and it continues in the unfed fly. Clearly then,

if yolk deposition is under hormonal control, the hormone must be activated some time during pupal development, and the taking of the bloodmeal bears no relation to its initial release into the haemolymph.

It has been shown that, after the bloodmeal has been taken, there is an increase in the rate of development of the egg follicles of *G. morsitans*. If the bloodmeal is delayed there is a corresponding delay in the acceleration of the follicular development. The size of the bloodmeal, however, does not affect the amount of growth occurring in the follicles, and there is no 'threshold' in the size of the bloodmeal (as has been demonstrated for some anautogenous mosquitoes) below which increased ovarian development does not occur. These results are additional evidence that the taking of the bloodmeal does not initiate or promote the release of a hormone by providing a stimulus in the form of abdominal distention. This is further illustrated, by the few females in which, after feeding, the blood fails to pass from the crop into the midgut. In these flies the blood remains undigested and the follicles do not grow any more than in the unfed females. Presumably, if secretion of a hormone was induced by the pressure of the blood on the body wall or crop, the follicles would have shown some increased activity.

Staining the fat bodies of unfed *G. morsitans* with Sudan IV on successive days of starvation has shown that fat reserves are practically exhausted by the 4th or 5th day after emergence (Saunders, unpublished). It is probable that the rate of follicular growth in unfed females is restricted by the gradual depletion of these, and other reserves, such as proteins necessary for the production of protein yolk. The reason for the increased development after feeding, therefore, is probably only a nutritional one, and even the smallest bloodmeal recorded in these experiments (8·0 % of the body weight of the fly) provided sufficient precursors for normal yolk synthesis.

VIII. SUMMARY

Five species of *Glossina* (*G. morsitans*, *G. pallidipes*, *G. austeni*, *G. brevipalpis* and *G. palpalis*) have ovaries containing two polytrophic ovarioles, each of which contains a single egg follicle. In all these species the right ovary is more highly developed than the left at emergence. This type of ovary is probably common to all species of *Glossina*.

The development of the egg follicles in *G. morsitans* pupae is described. It is shown that the cycle of development whereby eggs are produced alternately from right and left ovaries is established during pupal development and that some yolk deposition has occurred in follicle A (right ovary) and follicle C (left ovary) before the fly emerges from the puparium.

In *G. morsitans*, it is shown that the size of the egg follicles in newly emerged flies is correlated with the size (R.D.W.) of the fly, larger females having more yolk laid down in their follicles before emergence. This is probably correlated with the state of reserves in the fat body. It is also shown that there is a seasonal variation in follicle size in newly emerged flies, this variation is probably correlated with variations in temperature acting upon wild females during pregnancy and upon the pupae, as temperature is known to affect both size and fat content of wild tsetse flies.

The relationship between the size of the fly and the size of the follicles at emergence is also shown between different species. *G. brevipalpis*, which is a large fly, has, at emergence, large follicles with proportionately more yolk than those of *G. morsitans* and *G. pallidipes*, and *G. austeni*, a small fly, has small follicles with proportionately much less yolk. *G. brevipalpis* shows all four follicles descended from their germaria at emergence, whereas *G. austeni* shows only follicles A and C fully descended.

Some growth of the egg follicles occurs in unfed *G. morsitans*, even though reserves in the fat body become rapidly depleted by starvation. In fed flies the rate of growth of the follicles is accelerated. If the bloodmeal is delayed, the point at which this acceleration occurs is also delayed.

The size of the first bloodmeal taken by female *G. morsitans* increases as the fly gets older and more hungry. A single bloodmeal is not sufficient to allow for complete development of the first egg, although a few females which received two bloodmeals produced a mature egg in the right ovary. The size of the bloodmeal bears no relation to the amount of growth occurring in the follicles, and even the smallest bloodmeal recorded resulted in as much yolk deposition as the largest during the first few days. There is no 'threshold' in the size of the bloodmeal below which increased development of the follicles does not occur.

Because yolk deposition has already begun in the follicles before emergence and continues in the unfed fly, it is considered that, if yolk deposition is under some sort of hormonal control, the taking of the bloodmeal does not provide the stimulus for the release of the hormone. The reason for the increased follicular development in fed flies is probably a nutritional one.

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